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TECHNICAL PAPER

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Part 11 - Biological assessment of marine pollution — with particular reference to benthos

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PREPARATION OF THIS DOCUMENT

FAO participates in the implementation of the Long-term Programme for Pollution Monitoring and Research in the Mediterranean (MED POL) - Phase II, which is coordinated by the United Nations Environment Programme. In the framework of the MED POL programme, Mediterranean Institutions undertake research to study the ecosystem modifications in areas influenced by pollution. During Phase I of the programme a manual was developed entitled "Manual of Methods in Aquatic Environment Research, Part 8 - Ecological assessment of pollution effects" (FAO Fish.Tech.Pap (209), 1981), which aimed to contribute to the identification of the effects on marine life of pollutants from different sources. The manual was prepared by Professor J. Štirn.

The FAO/UNEP Meeting on the effects of pollution on marine ecosystems (Blanes, Spain, 7-11 October 1985) recommended that the above manual should be updated in order to maintain its usefulness as a guide to appropriate research techniques. Another conclusion of the meeting was that there is an urgent need for training in methods of data analysis. FAO/UNEP organised such training courses in cooperation with the Group of Experts on the Effects of Pollution of the Intergovernmental Oceanographic Commission (IOC/GEEP). Training workshops on the statistical treatment and interpretation of marine community data have so far been organised in Piran, Yugoslavia (1988), Athens, Greece (1989), Split, Yugoslavia (1990) and Alexandria, Egypt (1991).

The present manual was prepared by Professor A.D. McIntyre and Professor J.S. Gray and draws to a great extent on material from Part-8 of this series mentioned above and from the course lecture material. For this reason Professor J. Štirn has been retained in the authors.

Final editing and compilation was done by the staff of the FAO Fishery Resources and Environment Division, particularly Mr. G.P. Gabrielides. Ms V. Papapanagiotou was responsible for the typing.

The views expressed in the manual are those of the authors and do not necessarily represent the views of either FAO or UNEP.

DEFINITION OF MARINE POLLUTION

> Pollution of the marine environment means: "The introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) which results in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water and reduction of amenities".
>
> IMO/FAO/Unesco/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP)

Cover photo: Sampling of benthos in the Mediterranean using a Smith-McIntyre grab. Photograph by Dr V.A. Catsiki, National Centre for Marine Research, Athens, Greece

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SUMMARY

Chemical analysis, although valuable and necessary, does not provide all the information required in pollution assessments. Biological studies are of particular value in permitting a realistic assessment of pollution and they cover a wide range of possibilities. The present manual makes only cursory reference to the techniques used to study the sublethal toxic effects at the "individual" level of organisation or below since it is devoted to biological studies at community level and especially to the use of benthos. It describes how a benthic sampling programme should be designed so that the data collected can be best interpreted and evaluated. Information is provided for the collection and treatment of the samples as well as for the analysis of the data using statistical methods and computer software. Multivariate analysis techniques include hierarchical clustering, multi-dimensional scaling (MDS) ordination and principal components analysis (PCA).

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1. INTRODUCTION

There are many definitions of pollution, but the GESAMP formulation is widely accepted and constitutes part of the protocol of several international agreements and conventions. It states: "Pollution means the introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of seawater, and reduction of amenities" (Pravdic, 1981).

This definition first indicates that marine pollution arises from substances added to the sea by man. Although these substances cover the whole chemical spectrum they can be classified into a relatively small number of categories. These include:

nutrients
sewage
oil
metals
synthetic organic compounds
radionuclides
plastic litter
particulates

The definition secondly recognizes that pollution implies adverse effects on the environment. Nutrients, mainly nitrogen and phosphorus, whether derived from urban waste waters, from industrial discharges, from agricultural run-off or from natural weathering of the land, act as biostimulants, causing eutrophication - an enhancement of the growth of seaweeds and phytoplankton. This can lead to the development of unusual plankton blooms which may or may not be toxic but which on decay use up oxygen from the water with adverse consequences for fish and invertebrates. Sewage is another source of nutrients but in addition it contributes large amounts of organic matter which also causes deoxygenation. These effects of nutrients are found particularly in sheltered areas where water exchange with the open sea is restricted, and where there is considerable urbanization or industrialization, or where large rivers draining agricultural land reach the coast. The inner Adriatic is an obvious example, and also the Bay of Izmir and the Gulf of Lions. As well as affecting the plankton, eutrophication can alter the structure of benthic communities. Sewage presents an additional public health threat in that it carries pathogenic organisms that can cause disease in human beings from contamination of seafood and beaches. Oil enters the sea via operational discharges from ships and offshore installations, from accidents and from various coastal effluents. In large amounts it smothers habitats and organisms, and fresh oil has toxic components. It forms slicks on the sea surface damaging seabirds and marine mammals, while weathered oil washed ashore as tar balls reduces beach amenity. Although there have so far been no major oil spills in the Mediterranean, it is a busy shipping zone with many tanker routes, and drilling for oil and gas is currently conducted by eight countries in the region.

Metals are natural components of seawater and sediments, and as such are harmless to marine life, but they can build up to high concentrations as a result of Man's activities, as in mine tailings or industrial effluents, and may then represent a risk to human consumers of seafood. It is not always easy to partition the residues in animals between natural and anthropogenic sources. For example in some countries of the Mediterranean Sea (Algeria, Italy, Spain, Turkey and Yugoslavia) weathering of natural cinnabar deposits is thought to contribute to the relatively high level of mercury measured in some marine organisms, but in certain areas industrial effluents are also relevant.

Synthetic organic compounds, particularly pesticides (e.g. DDT) and certain industrial chemicals (e.g. PCBs) are now widely distributed in the environment. Being fat-soluble, persistent and largely non-biodegradable they accumulate in sediments and in the lipids of organisms. Their build-up in top predators, particularly marine mammals and birds, causes damage and their presence in seafood can make it unacceptable for human consumption. Some organometals such as TBT can be toxic at low concentrations, and chronic effects, including imposex in the gastropod Nucella, can be induced at very low concentrations.

Although radioactivity tends to arouse public concern, radiation from artificial radioactive substances is extremely low, reaching even the level of natural background in only a few localities. Radionuclides enter the sea from both natural and anthropogenic sources, but at concentrations which do not pose a threat to marine organisms. The main input from Man is in wastes and the largest quantity is derived from nuclear fuel reprocessing. This is not done on an industrial scale in the Mediterranean so most releases into that sea are from the 25 nuclear power stations which operate in four countries on the northern Mediterranean coast - France, Italy, Spain and Yugoslavia. Since most of those power stations are located alongside freshwater, their effluents are transported to the sea via river systems.

Plastic litter is a relatively new category of marine contaminant, but is causing concern. The synthetic materials now widely used for fishing nets, packaging straps and containers are buoyant and persistent. Discarded nets continue to trap animals by 'ghost' fishing, straps and rings encircle mammals, birds and fish, and plastic materials of all kinds accumulate on beaches.

A detailed evaluation of these pollutants and their effects on a global basis has been made by GESAMP (1990) and a related exercise involved a study of conditions in each of UNEP's Regional Seas areas. In the Mediterranean report it is stated that all confined or semi-confined localities adjacent to large urban centres appear to be in a state of progressive build-up of pollution as a result of anthropogenic release, while eutrophication is recorded as a significant problem.

Since pollution is related to the introduction of substances to the sea, a first approach to its measurement can be made by the chemical analyses of contaminants in the water, in the sediments and in the tissues of organisms. Such data on chemical concentrations can indicate actual or potential problems, especially if linked with information relating concentrations to effects, but by themselves chemical measurements do not constitute assessment of pollution. That assessment can properly be made only by observing in the field biological effects on the biota.

2. THE BIOLOGICAL APPROACH TO MARINE POLLUTION STUDIES

As indicated above, chemical analysis, although valuable and necessary, does not provide all the information required in pollution assessments. Indeed, it is not the concentrations of contaminants *per se* which are of concern, but rather the effects of these concentrations on organisms and on human health, and it is only by documenting these effects that the true significance of the chemical data can be defined. It is for this reason that biological effects studies are an essential component of any pollution assessment programme, and this was early recognized by FAO in the series of publications under the general heading "Manual of Methods in Aquatic Environment Research", some of which are referred to, as appropriate, below.

It is important, however, to clarify what is implied here by 'effect'. In a sense the accumulation of chemical residues in the tissues of organisms is an effect of the inputs and also the concentrations of contaminants in the water and sediments. But in terms of the present discussion a biological effect is more specific. It occurs when the organisms can be shown to react in some way to the contaminant. Knowledge of biological effects therefore provides information on the impact of the contaminant on the biota and since that impact may cause changes in population and communities of organisms, the acceptability of this can be considered, and the ultimate evaluation of the pollution can be made. Quite apart from providing this ultimate assessment, the use of the biological approach has certain practical advantages. One constraint of chemical analyses is that they are usually directed towards specific contaminants and will therefore omit any that are not on the suspect list, possibly missing a key toxic chemical. Studies of biological effects, on the other hand, pick up and integrate responses to the totality of chemicals in the water or sediments and so provide a comprehensive picture.

Biological studies are thus of particular value in permitting a realistic assessment of pollution, but they also assist in other ways. Since many animals,

particularly filter-feeders, accumulate chemicals from the water and sediments into their bodies (Portmann, 1976), they act as sentinels in providing an early warning of potential problems. Also, in the bioassay mode, test organisms can be used in experimental situations to appraise environmental quality (Reish and Oshida, 1986). Finally, at an even more finely focused level, organisms are used in what is usually referred to as toxicity tests, which include among other things, screening tests, tests to develop water quality standards, and legal tests, which are usually designed to determine LC50s (see for example, UNEP/FAO/IAEA, 1987a, b and c).

Biological effects studies proper cover a wide range of possibilities. An early workshop in this field (McIntyre and Pearce, 1980) identified some 50 relevant techniques, ranging through biochemistry, physiology, pathobiology, behaviour, genetics and finally population and community studies. These aspects are referred to in the following sections of this manual, but particular emphasis is given to the study of ecological effects.

3. BIOLOGICAL TECHNIQUES USED IN MARINE POLLUTION ASSESSMENT

This section considers briefly the approaches available and in current use to assess marine pollution. It focuses on techniques applied at and below the level of individual organisms, leaving treatment of the higher levels of organization (populations and communities) to sections 4 and 5.

3.1 Biological studies below the level of the individual organism

A stressor acting on an organism will produce a reaction and it should be possible to detect the effects at cellular and biochemical levels before these effects become obvious at the level of whole-animal physiological processes. A wide range of techniques has been proposed (Uthe et al., 1980; Moore and Lowe, 1985), of which some are listed below:

Blood chemistry studies, using blood serum assay techniques.
Adenylate energy charge determination, a measure of the metabolic energy available to an organism from the adenine nucleotide pool.
Metallothionein, which is the metal-binding protein induced in some organisms by the presence of heavy metals in the water.
Cytochrome P-450 system, which is induced by certain organic contaminants.
Lysosomal fragility, a study of the destabilisation of lysosomes (membrane-bound sacs containing enzymes, found within the cytoplasm of animals) by contaminants.
Steroid hormone metabolism studies.
Taurine:glycine measurement, involving a study of changes in the ratios of some free amino acids.

While these and other techniques have been studied in the laboratory, many of them would not be appropriate for application in the field on a routine basis, because of, for example technical problems and difficulties of interpretation (Lee et al., 1980). This prompted the Group of Experts on Effects of Pollutants (GEEP, sponsored by the Intergovernmental Oceanographic Commission of UNESCO, the International Maritime Organization and the United Nations Environment Programme) to set up a series of workshops where the most promising techniques were tested against each other along known pollution gradients. The tests were done 'blind' in that the research workers involved were not told where the samples had come from and had to rank the grades of pollution independently (Bayne et al., 1988). From this exercise, two reliable techniques have emerged.

First, where the pollution is an organohydrocarbon (PAH, PCB or related chemical) presence of the pollutant induces activity in the cytochrome P-450 system in both mussels (Mytilus edulis) and flounder (Platichthys flesus). By measurement of this enzyme it is possible to assess whether or not a likely pollutant has stressed the organism, (see Stegeman, 1980; Addison, 1984; Stegeman et al., 1988; Addison and Edwards, 1988 for methods). However, this approach must be used with caution, since at certain stages of the fishes' life cycle the P-450 system can be switched off, (eg. when egg formation is taking place) and false reading can be made. Also some evidence suggests that enzyme system can be induced only once; if a fish is left to recover fully after exposure to a stressor, a

second exposure may not result in further induction.

Second, heavy metals present in the water induce activity in metal-binding proteins (metallothioneins) in many marine organisms. Measurement of the presence of metallothioneins in Mytilus edulis (Viarengo et al., 1988) has been shown to be a reliable technique for the measurement of stress induction. Metallotheonein induction occurs in Polychaeta and some Crustacea and is likely to become a widespread tool in the future.

Biochemical techniques such as cytochrome (P-450) or metallothionein tests have the advantage that they are specific to a certain group of chemicals and indicate what detailed chemical analyses should be made in following up the investigation.

A number of other tests are available, such as alteration of cell membrane structure (Moore, 1988), and cytogenetic damage (Perry et al., 1988), but with the present state of their development, these do not seem as reliable as those mentioned above.

An approach using genetics is also relevant to studies below the level of the individual organism. In particular, the cells of fish, especially the eggs and early developmental stages are very sensitive to chromosome damage arising from contact with water-borne mutagens, and this can be used to measure the sublethal effects of sea-surface pollution. Longwell et al. (1980) provide details of the techniques and discuss the general approach.

In applying such methods it is important to make proper statistical analyses and Clarke and Green (1988) provide a useful detailed appraisal of appropriate statistical procedures.

Using the above techniques, stress can be measured at levels below that of an individual organism although it can be argued that they do no more than show that the organism's compensatory mechanism has been induced. For the technique to be relevant as a measure of biological effects rather than simply a means of indicating exposure to contamination, some detrimental impact on the growth, reproduction or survival of the organism must be demonstrated or at least some link shown with a physiological condition that does have such an impact. In this context, it is likely that prolonged induction of cytochrome P-450 activity or metallothionein will lead to effects at the population level, but data are not yet available to confirm this. Any effect should also be capable of showing a graded dose/response reaction.

3.2 Biological studies at the level of the individual organism

The biochemical techniques referred to above are responses to more or less specific stressors. The techniques described in this section are biological responses that indicate general pollution loadings, or otherwise make particular use of whole animals.

One obvious effect at the whole-animal level is morphological change, and a number of such changes linked to contaminants have been documented (ICES, 1978). These include damage to gill membranes exposed to zinc, gross changes in the liver caused by pesticides, and various skeletal anomalies involving the gill-rakers, opercular bones, cranial asymmetries, and vertebral column deformities in fish exposed to metals and organochlorines. Unfortunately many of the observations are derived from laboratory experiments in which very high levels of the contaminants were used. However, there are also field observations suggesting that fish, especially the younger stages, do exhibit such morphological changes when associated with pollution hotspots.

One group of morphological effects which is contaminant-specific is related to tributyl tin (TBT). This biocide is used as an antifoulant in marine paints, on shellfish traps and on the structures of fish farms. It causes is shell thickening in oysters and at very low concentrations it gives rise to imposex (females developing male characteristics) in some species of gastropods. These effects are easily detected by eye and are good biological indicators of TBT.

A further possible approach involving morphology is the use of disease-

related changes as an indication of pollution, and this has been advocated particularly in fish (Sinderman, 1983). To be of value in routine surveys the effect would need to be one which could be seen readily in the field, without the need for such detailed laboratory examination as the sectioning of tissues. In particular, fin erosion, skin ulceration and neoplasms, which occur on the surface of the fish, are obvious candidates.

Fin erosion is a non-specific disease but many records suggest that it is associated with degraded coastal or estuarine environments. Two types of fin erosion can be distinguished. One in demersal fish, probably related to direct contact with contaminated sediments, occurs mainly on the dorsal and anal fins, while another in pelagic species is more generalized but predominantly in the caudal fin. It is important not to confuse mechanical or net-damaged with fin erosion. True fin erosion is characterized by melanized and darkened tissues which are not found in net-damaged fish.

Epidermal ulcers have been observed in many fish species (Bucke and Watermann, 1988) and vary in size from small superficial lesions to large areas involving skeletal muscle and bone tissue. Ulcers may be caused by microorganisms such as vibrios or viruses but it has also been suggested that their prevalence is higher in polluted areas.

Neoplasms, or tumours, have been found in at least 60 marine species from a variety of habitats (Sinderman et al., 1980). These may occur on the skin as epidermal papillomas or carcinomas, or internally as tumours particularly in the liver. Most species with a higher prevalence of tumours in polluted waters dwell or feed on the bottom where the concentration of chemicals is usually highest.

The potential of using the prevalence of pathological conditions in marine animals as a tool in pollution monitoring does seem considerable. The main problem is that the outward expression of disease depends on highly complex ecosystem interactions and the separation of natural and anthropogenic impacts is extremely difficult. Research in this context is at present underway and in the meantime it is suggested that fish disease indices of pollution should be used mainly for the initial identification of hotspots and always as only one approach in a wider suite of studies. As referred to later, the use of fish disease in the context of populations is even more difficult.

There are three other types of approach involving the use of individual organisms - bioassays, behavioural studies, and the use of indicator species.

With bioassay tests, a single species is used to monitor the quality of water or sediment which is suspected of being contaminated. Such tests suitable for use in the Mediterranean sea have been described by Bellan (1981). One of the most intensively studied and widely tested bioassays is a physiological index of stress, scope for growth, in the mussel Mytilus edulis (Bayne, 1980; Bayne and Worral, 1980; Widdows and Johnson, 1988) which has been shown to be a reliable indicator of general pollution stress. In this test the energy costs of respiration and other physiological losses are compared with the food intake and thereby the physiological well-being of the individual assessed as the potential energy remaining for growth, the scope for growth. While the test is done on individual animals by taking replicates good statistical reliability has been obtained in this assay, (Widdows and Johnson, 1988). It may be argued that the test does not relate to the population as the response of individuals only is measured. However, the consequences at the population level of reduced scope for growth have been well tested, (Widdows, 1985; Koehn and Bayne, 1989) and if scope for growth is reduced over even relatively short periods of time then both fecundity and survival of the individual is also reduced and effects will clearly ensue at the population level.

While a wide range of organisms have been suggested for use as bioassay tests only a few can be reliably used. In addition to the scope for growth test in Mytilus, there are tests using oyster larvae (Woelke, 1967, 1968, 1972; Connor, 1972), echinoderm larvae (Kobayashi, 1971), Daphnia (Deneer et al., 1988) and Artemia (Abernethy and Mackay, 1986). The references quoted above provide details of the tests. The organisms are grown in culture under controlled conditions and performance of the organism is tested in the water to be assayed. For oyster and echinoderm larvae the percentage metamorphosis is recorded whereas the Daphnia and

Artemia test involves either percentage survival or growth. The tests grade the assayed water in relation to the control. Since in these tests the responses measured, i.e. percentage metamorphosis and percentage survival, are at the level of whole individuals it may be expected that the scope for growth test will be more sensitive. As yet no comparative experiments have been done.

Another group of organisms which has proved most useful in the bioassay mode is the hydroids, such as Campanulanias flexuosa. Hydroids are not of commercial importance, but they are easily handled in experiments because they are sessile, they are sensitive to stress, and they reproduce asexually so can be cultured as clones. They thus avoid some of the difficulties presented in working with larvae. The culture techniques and the operation of tests are described by Stebbing (1985).

Recently a new approach has been introduced, the sediment bioassay. Amphipods are known to be particularly sensitive to oil pollution and are therefore, appropriate to this type of study. The sediment is removed undisturbed from the natural habitat by a corer, amphipods are placed in the core, and the time taken to burrow or the percentage survival over 48 hrs is recorded (Chapman and Long, 1983; Long and Chapman, 1985). It is possible to rank the sediments in order of their degree of contamination.

The appeal of behavioural tests is undoubtedly high. It may be expected that organisms are able to detect a pollutant and initiate an avoidance response and thereby not be affected. Many techniques have been proposed, involving for example feeding, ventilation, heart rate, learning and shelter-building (Miller, 1980; Olla et al., 1980a). Yet surprisingly few behavioural responses have so far been adequately studied and/or quantified so that the techniques are not in general use. However, in concert with other approaches, the use of behaviour does seem to have much to offer (Olla et al., 1980b).

Another use of individual organisms is as indicator species. This involves recognition that the presence of an individual of a given species in a field sample may indicate a certain grade of pollution, and a few species have been proposed for this role. Capitella capitata is a small polychaete that occurs in high abundance under conditions of organic enrichment (Reish and Barnard, 1960). It is found almost world-wide where high levels of organic enrichment occur and was suggested (Reish, 1970) as a 'universal indicator of organic pollution'. There are however, problems with the use of such organisms. First, C. capitata occurs in high numbers under naturally disturbed conditions (Eagle and Rees, 1973; Grassle and Grassle, 1976a and b) and does not necessarily indicate organic enrichment. Second, C. capitata has been shown to be a complex of many sibling species (Grassle and Grassle, 1977) and thus may not in fact be a single cosmopolitan species. Third, and more seriously in the context of a universal indicator of organic pollution, it occurs extremely late in the sequence of organic pollution stress (Pearson and Rosenberg, 1978), and therefore indicates gross pollution rather than the first states of decline, which is often the object of pollution monitoring (Gray, 1981). By this stage the sediment has few species and usually smells strongly of H_2S, so it is much easier to use this property as an indicator than to search the sediment for small polychaetes. Thus, except to indicate extreme polluted conditions, use of an indicator species such as C. capitata is not recommended.

However, indicator species can be found which are sensitive to subtle environmental changes (Gray, 1989). These are not likely to be universal, but rather are specific for given localities.

Before leaving the examination of the use of individual organisms, mention should be made of the mussel-watch concept. It has long been recognized that filter-feeding animals, particularly bivalve molluscs, concentrate contaminants from the water, and Goldberg (1975) proposed that mussels could be used in a global monitoring programme. In the meantime, this approach is in widespread use at the regional level, for example, a national mussel watch programme has been underway since 1975 on both Atlantic and Mediterranean coasts of France, by which concentrations of several metals and organic contaminants are determined in the soft parts of mussels and oysters sampled on a quarterly basis (Claisse, 1989). It should be emphasized that this type of programme uses the animals merely as integrators of contaminants which are subsequently determined by chemical

analysis. However, if some of the biological effects techniques discussed above could be built into the exercise, then the value of mussel-watch programmes could be greatly expanded.

4. BIOLOGICAL STUDIES AT POPULATION LEVEL AND ABOVE

It can be argued that although effects may be shown at the level of the individual or below, in dealing with organisms other than Man it is only when such effects have consequences at the population and community levels that the pollution has any real significance (McIntyre and Pearce, 1980). Yet it is difficult to measure effects at these higher levels, and in particular to detect a statistically significant change in population size which could be related unequivocally to pollution. Indeed, there are almost no life-table data available on naturally occurring non-commercial marine species so that little is known on changes in survivorship (l_x) or mortality (m_x) in response to pollutants. Linked to studies of, for example, scope for growth, field studies of life-table data could be a promising area of research.

Most field studies of pollution effects at the population level have been directed to the plankton or the benthos. Fish may seem to offer a promising approach, in view of the large amount of data on commercial species. However the major impact on the stocks is undoubtedly due to fisheries exploitation, and this makes it difficult to identify stock changes that can be attributed to pollution. Thus, although a great deal of information is available from commercial fishery research and statistics, it cannot be said that fish are particularly attractive for pollution assessment studies at the population level, although they are much used for chemical monitoring.

4.1 The use of plankton

Plankton, on the other hand, is a primary recipient and target for the great majority of polluting inputs. Reference has been made to the use of zooplankton (oyster and echinoderm larvae) in bioassay experiments, and there are descriptions of the employment of phytoplankton in rotating dialysis bags suspended from buoys *in situ* for marine pollution monitoring (Jensen, 1980).

There are also studies of plankton in mesocosms - large enclosures which can be treated with a variety of contaminants and the populations studied over periods of at least several months, thus introducing ecological realism to the experiment. Mesocosms can range in size from a few cubic meters to as much as 1,300 m^3 and the design can be varied to suit a diversity of facilities, requirements and costs. This approach may be recommended as a useful adjunct to other studies. It can help in the interpretation of field observations and in suggesting new ways of looking at problems. The use of mesocosms is reviewed by Grice and Reeve (1982) and Kuiper and Gamble (1988).

However, direct field studies of effects on plankton are a different matter. Even although the initial release of contaminants may be in the pelagic zone, dilution and dispersion tend to be rapid there, and organisms will be carried away from fixed sources of input. One consequence of this dilution and transport is that impact on plankton is likely to be substantially less than on sessile benthic organisms.

This does not apply in areas where water exchange with the open sea is restricted and flushing is poor. In such conditions effects on plankton may be expected and indeed are well documented, particularly in relation to excessive input of nutrients (Relevante *et al.*, 1985). This results initially in enhanced plant growth, culminating in unusual plankton blooms (sometimes toxic), and in a change in the structure of the phytoplankton community, with smaller flagellates replacing larger diatoms as the main components. Changes in the zooplankton may also occur. Benović *et al.* (1987), working in the northern Adriatic, recorded changes in the hydromedusan faunas, with significant reduction in those components (anthomedusae and leptomedusae) which have bottom-dwelling (hydroid) phases in their life cycle. This is correlated with reduced oxygen in bottom waters associated with eutrophication. Thus, changes in the structure of plankton communities in certain areas can be good indicators of eutrophication.

But, in general, impacts of pollution in the water column are best studied in relation to the nature of the input. First, in the context of point sources of input, and second when the input is diffuse. Point source input is particularly relevant to plankton in the case of ships dumping at sea, and releasing large amounts of material, usually industrial wastes or sewage sludge, into surface waters. For example, studies at an ocean dump site for pharmaceutical wastes off Puerto Rico showed an immediate change in the phytoplankton community structure at the site, but no persistent long-term changes were observed (Murphy et al., 1983). Further, looking at zooplankton, a survey of industrial wastes discharged in deep water off the east coast of the United States (Capuzzo and Lancaster, 1985) showed that only a small percentage of the community was affected, and that long-term consequences to the zooplankton populations were negligible. Another situation relevant to plankton is an oil spill producing a major surface slick. There are a number of studies in which the plankton under such slicks have been examined. Under a fresh slick from a large spill in the most extreme conditions, organisms and pelagic eggs may be coated with oil and there will be toxicity effects. However recovery can be complete in a matter of days (Davenport, 1982). If there are no significant effects from these discharges, it is most unlikely that any impact from diffuse inputs could be detected. For these reasons plankton is not considered further in the manual.

5. THE USE OF BENTHOS

As suggested by the discussion above the most suitable programme for examining the effects of pollutants on marine systems will be an analysis of effects on benthic assemblages. Such assemblages are widely used because:

a. the organisms are largely sessile and must therefore tolerate the pollution or die.

b. the assemblage integrates effects of pollutants over time.

c. a wide range of taxonomic diversity exists with upwards of 100 species per sample for both macrofauna and meiofauna.

d. there are many examples of such assemblages showing effects of pollutants [e.g. oil pollution (Davies et al., 1984; Reiersen et al., 1989; Gray et al., 1990); organic enrichment (Pearson and Rosenberg, 1978; Mirza and Gray, 1981; Bellan, 1985; Warwick et al., 1987; Bellan and Bourcier 1988, 1990); tannery effluent (Zenetos and Papathanassiou, 1989).

5.1 Choice of sample site

In the case of point-source pollution sampling will be done in the vicinity of the discharge and although the details of the sampling programme will require attention, the selection of the site in general will be obvious. However, when studying diffuse sources, careful site selection is essential, since a major problem in analyzing the effect of pollutants at the population level and above is the difficulty of separating 'nuisance' environmental variables from pollutant effects. Depth variability and grain size variation are typical 'nuisance'variables. Where possible such variables should be held constant by sampling at constant depth or within a narrow range of grain size. If this is not possible it is important to match, for their physical variables, control sites with impacted sites so that valid statistical comparisons are possible. A sample site for the study of non-point source contamination is often difficult to define. The objective should be to demonstrate whether a delimited geographical location is more impacted than a control site. A site may, for example, be defined as a 100x100m area from within which adequate replicates can be taken.

5.2 Acquisition of data and preliminary investigations

The design of programmes for quantitative and representative sampling of benthos is always a difficult task, and the more information there is on a selected area before planning, the better the sampling programmes that can be designed. The following information is needed for proper planning of quantitative benthic studies.

(i) Bathymetric and geomorphological data for the investigated area which may be available from existing documents, compiled into a basic chart of the area. If such a compilation is not adequate for the presentation of major geomorphological formations of the submersal coastal slopes and of the plain sea bottom, additional echo-soundings along critical transects in deeper waters and orientative mapping by divers for coastal hard bottoms should be done.

(ii) Sedimentological data from all available sources, including navigation charts, plotted in the form of convenient histograms on the basic map. By simple interpolations a map of the topographical distribution of the major sedimentological types within the investigated area can be made. This information, combined with the knowledge on the distribution of distinct types of water masses within the investigated area constitutes one of the most important elements in planning quantitative sampling programmes. Therefore, it is advisable to complete sedimentological studies of the investigated area (if such data are not already available) before the final setting-up of benthic sampling programmes, although both samplings are usually carried out at the same time for practical and economic reasons.

(iii) Oceanographic data are essential on distribution of water masses and their movements as well as the trophic conditions in the pelagic environment of investigated areas.

(iv) Any known inputs of pollutants should be examined with a view to charting their spatial distribution within the investigated area. This has to be done in order to select a suitable area for study and to identify its extension and limits. In some cases information is provided by the data on, for example, spatial distribution of coliforms and detergents, which can serve as tracers for the marine distribution of sewage and for the majority of mixed industrial effluents. For industrial effluents which do not contain these tracers, the detection of effluent distributions will require analyses of specific communities. The most useful information generally and for the latter cases in particular, is provided by a knowledge of prevailing currents and other movements of water masses within the investigated area, indicating most probable distribution of pollutants.

(v) Qualitative data on types of benthic communities and their biota, alone or together with the above information, form the basis for the design of sampling programmes. Therefore all existing information should be compiled and brought up-to-date by preliminary benthic investigations, carried out by qualitative dredging on soft bottoms and by direct diving observations and collecting on hard bottoms. Observations and underwater photographs made by divers are also extremely useful. The divers' information can be supplemented by the use of remote cameras or underwater television equipment, and indeed for deeper bottoms (over 50 m), where divers cannot work, these will be the only photographic opportunities.

5.3 Design of a benthic sampling programme

The ultimate objective of the programme will be to detect any change in the benthos, spatial or temporal, in addition to that due to natural variability, and to attribute the change to its cause.

If there is a point source such as an effluent pipe, a dumping vessel or an oil platform, and therefore a relatively small area of impact with a strong gradient, then the preliminary survey will suggest roughly the direction and extent of any dispersion, and will indicate the most appropriate layout of sampling stations. This may be in the form of a grid, covering the area or in lines arranged to sample the range of the gradient.

On the other hand, if diffuse inputs are being studied and therefore an

extensive area is involved, then as a result of the preparatory work, an initial survey should be carried out to map the extent of the various types of habitat within the area. It is obvious that sampling methods will vary with habitat, the optimum approach for sea-grass beds will be different from that for soft sediments and different again for rocky situations.

Within each relatively homogeneous habitat it is recommended that a stratified random sampling programme be used. In such a programme the expectation is that the fauna or flora responds to key environmental variables. For example in a sea-grass bed, depth may be an important variable so that sampling should be stratified for depth. In the case of soft sediments it is known that grain size has a strong influence on benthic macro- and meiofauna so the stratification should be for grain size variations. The area is mapped and the various grain size distributions plotted in.

An example follows, taken from Elliott (1971), showing how to plan a stratified random sampling programme for a benthic survey. It is planned to sample an area of 200 m^2 with a grab taking an area of 0.05 m^2. Potentially therefore, there are 200/0.05 = 4,000 sampling units within the area. A preliminary survey shows that the bottom is very heterogeneous. Since it is known that grain size variations could be important in determining species distributions, the sediment is mapped. Sampling should ultimately be done with equal intensity on each type of bottom. This is called proportional allocation of samples. Here an even coverage of 10% is given to each area i.e. 40 samples total, a not unreasonable number.

The preliminary survey shows that gravel (n1) covers 1000 sampling units, coarse sand (n2) 500, sand (n3) 1500, fine sand (n4) 800 and mud (n5) 200, totalling 4,000 sampling units.

The 40 samples are then allocated in proportion:

n1 = 1000 * 40/4000 = 10 samples
n2 = 500 * 40/4000 = 5 samples
n3 = 1500 * 40/4000 = 15 samples
n4 = 800 * 40/4000 = 8 samples
n5 = 200 * 40/4000 = 2 samples

As to the placement of samples, ideally the whole area is divided up and each potential sampling unit is given a number, picked out from a table of random numbers.

Another method of allocating samples within a stratified random approach is optimal allocation, which provides for more samples to be taken where there is high variability. An example of such an approach is shown in Appendix 1.

5.3.1 Size and number of replicate samples per site

The general rule for sampling is that many small samples are better than few large ones. The reasons for this are that with many small samples a greater coverage of the sample site is achieved, a better estimate of the spatial dispersion of species is obtained and there are a greater number of degrees of freedom for statistical analyses. However, the size of sample should not be reduced to such an extent that so-called 'edge effects' (disturbance of the sample by the edge of the sampler) dominate. More often than not the size of sample is prescribed by the type and size of the gear available. It should not be assumed however, that a particular grab (or plankton net) is the appropriate size for a given population simply because it is available. Most grabs were developed as fractions of $1m^2$ and are not necessarily appropriate for all situations. For a general guide to marine examples for determining sample size the reader is referred to Elliott (1971) and Venrick (1978).

For statistical analyses an adequate number of replicate samples must be taken. In practice this means a minimum of 2-3 replicates and with benthic sediment samples 5-10 replicate samples is common.

There are a number of more or less objective criteria for determining the number of replicates required. In sampling sediments an estimate of the total

number of species within a given area may be needed. A species-area curve is then plotted of the cumulative number of species against number of samples (Figure 1). From the shape of the curve an estimate can be made of the number of replicates necessary to obtain an acceptable % of the total number of species. In this example for C_4 five samples will give approximately 70% of the total number of species.

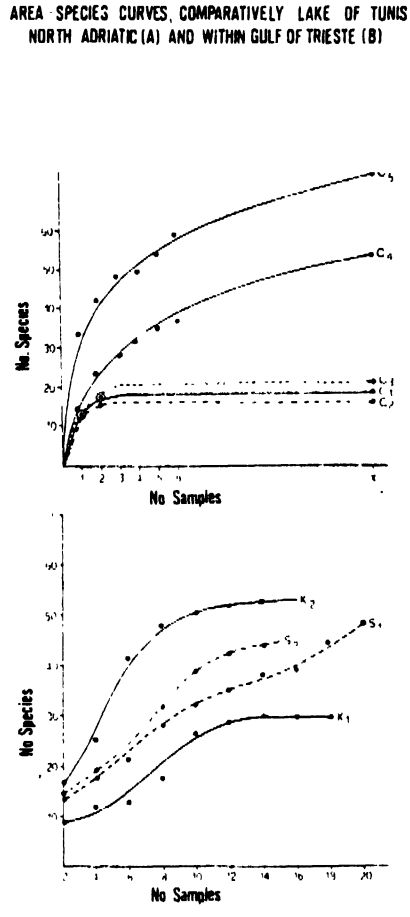

Figure 1. Various types of area/species curves from "normal" (curves C_4, C_5, S_1) and pollution or estuarine "stress communities" (curves C_1-C_3, K_1, K_2 and S_2). (From Stirn et al., 1975, with kind permission of Pergamon Press Ltd., Oxford)

It may also be useful to know the number of replicate samples needed where the primary aim is a study of the population dynamics of one single species. The interest is in estimates of the population mean number and its variance. A simple method to determine the number of samples required is to take five samples and calculate the mean and variance. Take five more and calculate the mean and variance for all ten samples and repeat until the mean and variance are stable. The minimum number of samples which give a stable mean and variance should be used.

A more elaborate method is to decide on an acceptable error of the estimate of the population mean and use this to calculate number of replicates. An example is shown in Appendix 2.

In cases where the interest is in obtaining population estimates for a given

species the number of samples to be taken is often large. For example in a study of the bivalve Mya arenaria in the Oslofjord, using the above formula, 30-35 samples were taken on each sampling occasion and the required number of samples was calculated in the field (Winther and Gray, 1985).

In conclusion, the number of samples to be taken depends greatly on the question asked. If the interest is related to the number of species, or species diversity, then samples must be taken to obtain the maximal number of species. For studies on the dynamics of one or a few species the formula above may be used.

5.3.2 Temporal sampling frequencies

The frequency of sampling depends on the question being asked and on the amount of information already available. If there is no background knowledge of the area under study then samples at regular intervals over a year are needed to ascertain the seasonal changes in the assemblage. In practice this usually means sampling monthly. Knowledge of seasonal changes can reduce the sampling effort. In pollution studies it is often important to know how the assemblage changes over time in response to the pollution load. It may be a waste of effort to sample seasonally and measure recruitment of juveniles of species which will die later in the season. If the interest is in changes from year to year then sampling at seasons where the lowest abundances occur (often winter) would be optimal. Figure 2 shows data for a subtidal hard bottom species sampled seasonally for a number of years. Clearly for this species the same trends will be in evidence whether sampling is at times of minimal abundance (winter), maximal abundance (summer), or takes the mean of all seasons. So a rationalized programme would sample once per year in mid-winter if the interest is in year-to-year fluctuations.

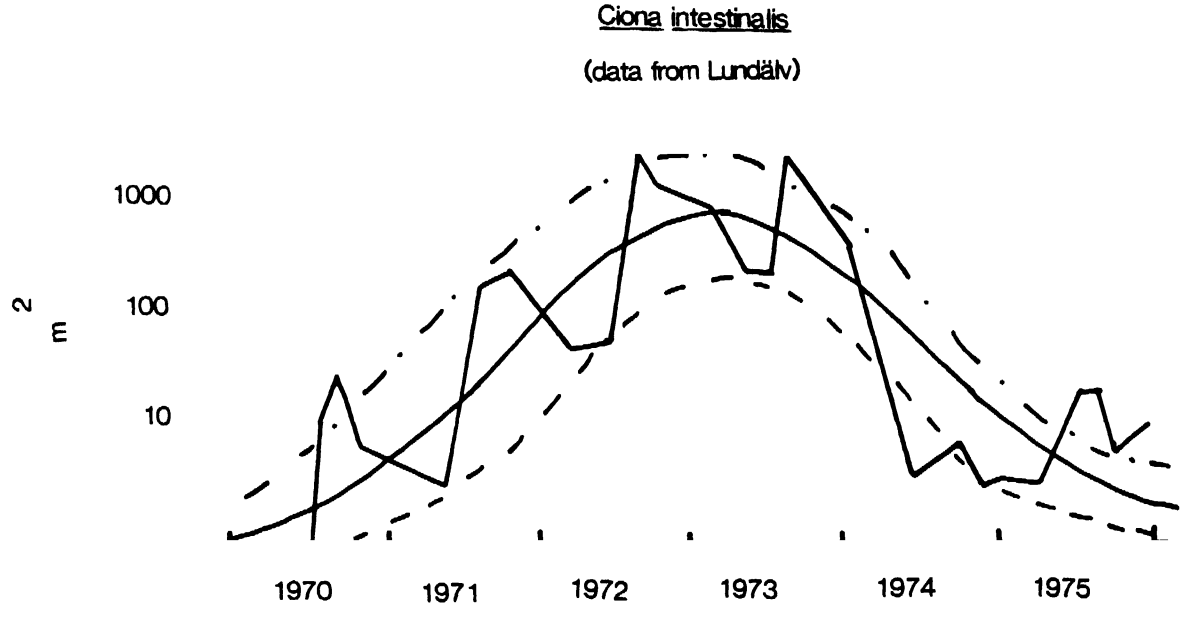

Figure 2. Ciona intestinalis populations in Gullmarfjord, Sweden studied using stereophotographic methods. Jagged solid line: actual data measured approximately monthly. Smooth solid line: mean abundance. Broken line: minimal abundance. Broken and dotted line: maximal abundance. (Data from Lundälv, unpubl.)

5.4 Sampling and processing methods

Decisions on the methodology and equipment will depend on the aims of each specific exercise, on the nature of the habitat involved and on the staff and facilities available. Each individual scientist tends to have his or her own preference for equipment and procedures, and any given laboratory may have its own traditional approach, determined partly by its research history. As a result there is a great diversity of methodology and for any single study it is usually

appropriate that the investigator should select the approach with which he is best equipped and most comfortable. However, in the context of collaborative or regional sampling programmes or international surveys, the use of standard, agreed methodology is important if results from different laboratories are to be linked and compared. This is discussed later in more detail.

5.4.1 Intertidal sampling

A few parts of the Mediterranean Sea have regular tides (eg the Gulf of Gabes, upper Adriatic) with an average vertical amplitude of 80 cm, but for the most part tidal oscillations are extremely small, with vertical amplitudes of less than 40 cm, resulting in narrow intertidal areas. Indeed, in the Mediterranean vertical divisions in the marine environment are usually described in terms of "zones". The region above the level not constantly covered by the sea is referred to as the mid-littoral zone (divided into an upper sub-zone which is wetted only by waves and a lower sub-zone, covered at high tide and wetted by waves only when the tide is low) and the suppralittoral zone which is wetted only by spray and where immersion is exceptional (Augier, 1982). The species composition of these communities varies considerably in different parts of the Mediterranean. The major components are calcareous and soft red algae, some brown and green algae, along with intertidal species of, for example, molluscs and cirripeds. Where soft shores occur the deposit is largely of sand, and the macrofauna (mainly some burrowing polychaetes and amphipods) is low in both species diversity and population density. The intertidal zone, narrow though it is, is extensively used for recreation and tourism and is highly sensitive to contamination from the land, so it will be important to study it in the context of pollution.

On rocky shores, the great diversity of habitat, including pools, exposed rock, sheltered crevices and the undersides of stones, makes it difficult to collect samples which are adequately representative of wider areas, and separate estimates for each distinct habitat will be required. A square frame of wire can be useful in defining the study or sampling area, and organisms either counted or collected, and the percentage of the area covered by organisms estimated. Frames of 1 m^2 or 0.25 m^2 are frequently used, but on very irregular surfaces or for small organisms, frames of 316 x 316 mm (0.1 m^2) are more suitable. For small species such as barnacles which may occur close together in large numbers, even smaller areas are appropriate, and a piece of thick perspex 100 x 100 mm (0.01 m^2), etched with a grid of 10 mm squares facilitates counting. Counting or sampling is usually done along traverses with stations at regular intervals or at specific tidal heights.

On soft shores representative sampling is easier. Undisturbed cores of sediments can be collected by pushing tubes of plastic or metal into the sand, the diameter of the tube being chosen depending on the volume or depth of sample required. For larger samples a square frame of sheet metal, usually enclosing an area of 0.1 m^2 or 0.25 m^2 is driven into the sand and the deposit dug out to the required depth. The sand is then sieved through a mesh of 0.5 or 1.0 mm depending on the size of the particles and the nature of the results required. A detailed discussion of intertidal methods for both hard and soft shores is given in Price et al. (1980).

5.4.2 Subtidal sampling: Ships and shipboard equipment

For shallow water work in the infralittoral zone close to the coast small boats of 7-10 m length, with 20 to 30 hp engines are usually suitable and can operate light gear-dredges, beam trawls and even small corers and grabs, which can be hauled manually using ropes. Positions can be determined from marks on the land.

For most subtidal work, however, larger ships are required that are fitted with winches suitable for hauling wire ropes for dredging and wire for grabs and corers which are operated vertically. For trawling and dredging, warps of from 12 to 24 mm diameter are used, and on the shelf a length of warp about 2½-3 times the depth of water is usually used. When operating grabs of 100-150 kg, galvanized steel wire of 6-8 mm diameter is appropriate.

If a vessel built for research is available the required equipment will be on hand, but if it is necessary to charter another type of vessel, it is important

to ensure that cranes or booms and winches with appropriate wire are available, that relevant navigational facilities are fitted and that there is a suitable echosounder. A convenient source of running seawater on deck is required and there should be sufficient free deck space for handling samples as well as bench space under cover for processing, and space for storage.

5.4.3 Subtidal sampling on hard bottoms

Rocky bottoms present the most difficult problems for remote sampling. If not too steep or uneven they can sometimes be surveyed using a heavy duty dredge which may provide at least qualitative samples. For example, a naturalist's or rectangular dredge with a 12 mm nylon bag is suitable. Due to rocky or encrusting irregularities of hard bottoms, the cables and other gear connected to the dredge must be strong enough to hold forces up to 1000 kg. The dredge is equipped with a weak link (Figure 3) consisting of several turns of twine (for heavy duty, three turns of 8 mm manila rope) which breaks if the dredge is anchored or stuck between rocks, allowing the arms to open and free it.

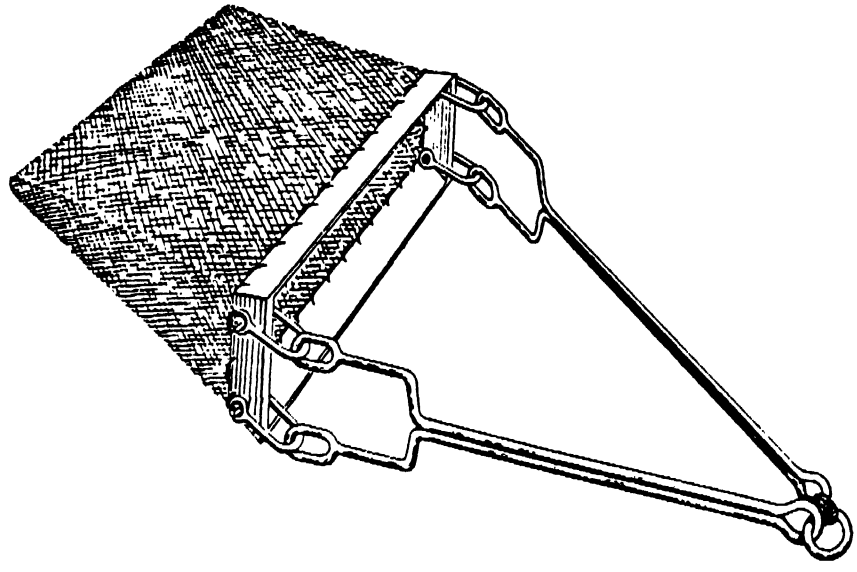

Figure 3. Naturalist's dredge. Note the position of a "weak link". (From Holme and McIntyre, 1984, with kind permission of the International Biological Programme, London)

However, in general, remote sampling gear such as dredges, grabs or corers are not appropriate for hard ground. Underwater photographic or television cameras lowered from a ship can provide useful information, but the most suitable approach is the use of diving. This is dealt with in detail in Section 5.4.5.2.

5.4.4 Subtidal sampling on soft bottoms

5.4.4.1 Qualitative and semi-quantitative sampling

For subtidal sampling on soft bottoms a wide variety of equipment is available, allowing for a diversity of requirements. Towed gear such as dredges and trawls provide qualitative and sometimes even semi-quantitative material. Beam trawls are available in several different forms (Figure 4) but basically they all consist of a long net, the mouth of which is held open by a rigid beam with metal runners at each end. The lower leading edge of the net is usually weighted or attached to a chain which curves back behind the upper leading edge of the net attached to the beam, thus preventing the escape upwards of mobile organisms disturbed by the ground rope. The Agassiz trawl is essentially a symmetrical beam trawl without a leading edge and with the net attached to two metal shoes so that it can be used either side up. It is thus suitable for deep water sampling when the landing of the gear on the bottom is difficult to control.

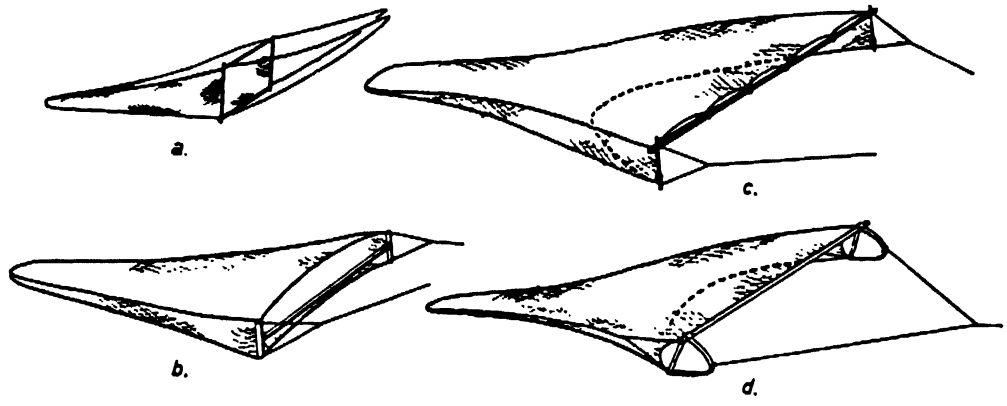

Figure 4. Beam trawls for qualitative sampling. (a) towed stow net; (b) 'Keitel' of Curishe and Frische Haff (both are lagoons on the southern coast of the baltic); (c) Japanese beamtrawl; (d) modern European beamtrawl for shrimps.(From von Brandt, 1981, with kind permission of the author)

Dredges, which are simply heavy metal frames fitted with a bag or coarse net, have already been discussed for hard bottom sampling and are also suitable for soft sediments. They are easily made in a wide range of sizes and weights, and the collecting bag can also be adapted in design and construction to meet specific sampling needs. Light dredges can be hand-hauled from small boats, while for deeper water, heavier gear is required for operation by winches. On silt-clay grounds on the shelf or beyond, the anchor dredge of Sanders can provide large samples. As shown in Figure 5, it has two angled digging edges with a heavy horizontal plate between them which determines the digging depth. Another semi-quantitative instrument widely used in the Mediterranean is the Charcot dredge (Picard, 1965).

The otter trawl, as used by commercial fishermen, has the net spread open by two otter boards. The trawl is shot on twin warps either over the side or from the stern and is reeled in using a double-barrelled winch. A variety of trawl gears is now available and a considerable range of ancillary equipment makes trawling, although still not fully quantitative, a highly sophisticated operation and one which, particularly at shooting or hauling, should be done by experienced specialists.

While trawls and dredges can be made at least semi-quantitative by standardizing as much as possible the condition and duration of towing, they are basically qualitative sampling devices. However, being mostly relatively simple gears, they can often be used in circumstances when more sophisticated equipment is not appropriate, and are invaluable in providing an initial indication of the general nature of a habitat and its fauna and flora.

5.4.4.2 Quantitative sampling

Reasonably quantitative sampling on soft bottoms is possible, and grabs and corers are appropriate, - instruments which are lowered vertically on a warp, the grab having jaws which 'bite' out a volume of sediment, while corers penetrate the deposit and on being hauled, carry a plug of sediment with them.

(a) **Macrobenthic infauna.** Many methods and types of sampling gear are available and a comprehensive review is given by Eleftheriou and Holme (1984) who list and discuss more than 20 samplers. Some of these are highly sophisticated and require specialist back-up equipment or the assistance of divers and can be most effective if such support is available. The ultimate choice of sampler will depend on the detailed requirements of the exercise as well as on the working conditions and the nature of the sediment. It is advisable to use the simplest gear that will provide a satisfactory sample and for general collecting and survey work one of

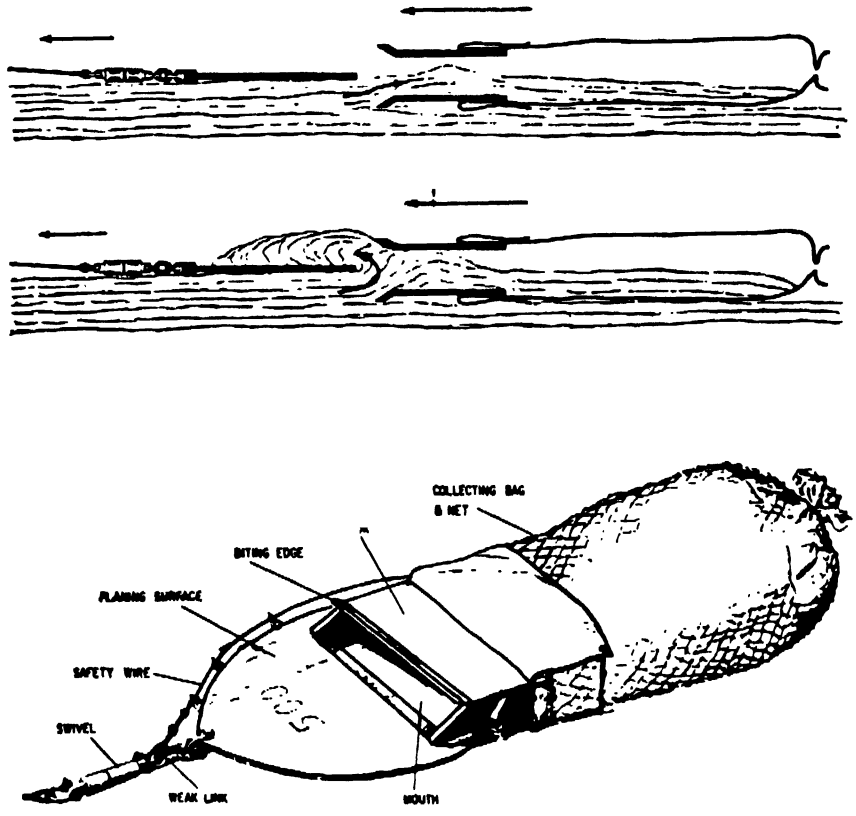

Figure 5. Anchor dredge Sanders type (From Sanders et al., 1965, with kind permission of Pergamon Press, Oxford)

the several developments of the original Petersen grab - a weighted hinged bucket, is recommended. The Van Veen model is virtually a Petersen grab with arms. The Smith-McIntyre added a frame, springs to drive the jaws into the sediment, and trigger plates at opposite corners which ensure that the grab would not activate until it was sitting squarely on the bottom. The Day grab is a simpler version of the latter and is now widely used. These grabs usually cover a surface area of 0.1 m^2 (although smaller and larger versions are available) and weigh around 30 kg empty. On soft mud a light grab will penetrate well but on hard-packed sand it is important that the gear should be heavy enough to bite deeply into the sediment rather than simply scrape the surface on lift-off. The digging depth of the instrument should be at least 10 cm. In this context the volume of sediment in a grab should always be measured, either by a graduated stick or by transferring it to a marked bucket. Less than about 4 litres of sediment in a 0.1 m^2 grab indicates poor penetration, and if the volume of sediment is too low the sample should be rejected. The handling of the winch which operates the grab is also important. The wire should be kept as vertical as possible to ensure that the instrument is set down and lifted up at right-angles to the bottom, and this means that working in bad weather is difficult or impossible. It is often useful to stop the winch for a moment just before the grab hits the bottom so that the setting down is done as gently as possible to lessen the shock wave and reduce the washing away of sediment. Hauling should be commenced as soon as the grab is properly settled on the bottom, since any delay will increase the wire angle if the ship is drifting, and the instrument will be hauled out obliquely giving a reduced sample. It is important to haul very slowly until the sampler has left the bottom.

In contrast to the principle of the grab, a corer is an instrument in the form of a tube which penetrates the bottom usually by its own weight, and retains a plug of sediment. Corers are usually smaller than grabs and are discussed below

in the context of meiobenthos, but one type of corer appropriate to macrofauna is the box sampler, which is considered here. This was first described by Reineck (1958) and consists of a rectangular corer supported in a metal frame. The corer penetrates the bottom assisted as necessary by added weights, and a hinged cutting arm swings down when the warp is hauled, closing the bottom of the tube and retaining the sample. The instrument samples an area of 20 x 30 cm to a maximum depth of 45 cm and weighs 750 kg in use. There have been several modifications of this design, such as the spade corer of Hessler and Jumars (1974) which covers an area of 0.25 m^2. These instruments are most satisfactory in that they provide relatively undisturbed samples to considerable depths, and the boxes containing the cores can be removed for convenient study in the laboratory. Unfortunately, box corers are not only expensive but also extremely heavy and very large, so that they are difficult to work and require the facilities of a large ship.

(b) **Meiobenthos.** Because of their small size and high density, organisms of the meiobenthos are best collected in small samples of sediment. This can be done by subsampling from a grab haul but such a procedure is unsatisfactory for several reasons, particularly because the down-wash of the grab will have disturbed the surface sediments where the meiobenthos is often richest, and because the closing of the grab and its passage to the surface will further disturb the contents so that a representative sample will be difficult to obtain. Subsampling from a grab should therefore be a last resort. A box sampler can provide a better possibility for subsampling, but even here some of the objections listed for grabs are likely to apply.

The most satisfactory approach is to collect a small sample dedicated to meiofauna, and the ideal instrument for this is a corer. The best samples are taken by divers operating a core tube manually but as indicated in section 6.4.5 there are significant constraints on diving work. The simplest remote corer is still an open barrel instrument such as that designed by Moore and Neill (1930). This collects good samples from muddy bottoms, especially if it penetrates a layer of clay which acts as a plug to prevent the mud core sliding out. Often, however, and always on sand, some type of core retainer must be fitted to close the lower end of the tube. A more sophisticated instrument is described by Craib (1965). This is a tube of 5-7 cm diameter which is mounted on a frame. When the frame comes to rest on the bottom the tube is forced slowly into the sediment by weights, controlled by a hydraulic damper, ensuring minimum disturbance of the light surface layer of the deposit. Samples of 15 cm in length are obtained and a closing device ensures that even samples from hard-packed sand are retained. The apparatus, weighing 44 kg, can be handled from a small boat. A larger version, employing multiple core tubes for use in deep water has been developed by P.R.O. Barnett of the Scottish Marine Biological Association and is described in Holme and McIntyre (1984). The advantage of cores for meiofauna is that the whole sample can be examined (split into layers if desired to study vertical distribution of the fauna) without resort to subsampling which can cause errors. For this reason the diameter and length of the core should be carefully selected so that the effort in counting and processing the fauna is not too great. It has been found that cores of 2-4 cm diameter are satisfactory for most purposes. In areas of very dense population smaller diameters may be appropriate but if the tube is too narrow, difficulties will be encountered in adequately collecting the surface fauna (McIntyre, 1971).

5.4.5 Other techniques

In several of the paragraphs above, reference has been made to special techniques such as diving and underwater photography, which are at least useful additions to a programme and in some cases the best or even the only adequate approach. These are discussed in more detail below.

5.4.5.1 Diving

In shallow water, diving using self-contained underwater breathing apparatus (SCUBA) allows detailed direct observations to be made, notes and photographs to be taken, and *in situ* experiments to be set up and operated. It also permits the collection of specimens and samples by hand, thus giving access to material from habitats, such as rocky bottoms, where the use of remote sampling gear is difficult or impossible. A comprehensive review is given by Gamble (1984).

In view of the value of this approach, it is important to recognize the limitations. Diving can be extremely dangerous and it is essential that those involved should be adequately trained, be fully aware of the safety requirements and should observe the rules, as set out in the manuals and codes available (eg NOAA, 1979 and Underwater Association for Scientific Research Ltd. 1979). When working in sewage-polluted waters, divers should be vaccinated appropriately for protection against pathogens likely to be encountered. Divers should operate as part of an experienced team and should never work alone, indeed in some countries this is illegal. The main restriction to SCUBA diving is depth. The time of a dive on air to deeper than 10 m is limited by decompression considerations and nitrogen narcosis restricts air diving to about 50 m. Temperature also limits the time a diver can spend underwater and visibility can be another constraint, since suspended particles, either biogenic or inorganic, can reduce transparency and light to virtually zero.

Divers can work free in the water, or can operate from towed gear or driven vehicles such as submersibles. The simplest gear is the underwater sledge which can be towed, for example, along with a trawl.

Whenever possible the diver should log his observations *in situ* to prevent inaccuracies which might arise from relying on memory for later recording. A camera is obviously of great assistance and its use is discussed below. More immediate recording can be done underwater by making notes on plastic board using a graphite pencil. For convenience the pencil can be attached to the board by a cord and the board should be fitted with a wrist strap. The use of speech is also possible and divers can either talk into a cassette tape recorder in a waterproof housing strapped to the aqualung cylinder, or communicate directly with the surface vessel. In this context bone conduction microphones held tightly against the diver's skull by the suit hood are appropriate (Main and Sangster, 1978). However, modification of the usual diver's mouthpiece is required if understandable speech is to be produced, and some practice is required in interpretation. In skilled diving teams the hand signal speech of dumb persons can provide a useful means of communication.

In spite of the limitations already discussed the diver, in collecting samples, has certain major advantages over remote gear used from ships. In very turbid areas over fine sediments it may be necessary to operate by touch, but in general the diver can examine the substratum and determine the exact location of his sample; he can see the diversity of the bottom and will know how representative his sample is; he can observe the reactions of organisms and be aware of the escape of mobile species. The most straightforward sampling operation is simply the picking up of flora and fauna by hand, using hammer and forceps when necessary. Larger specimens can be placed in prelabelled mesh sacs, polythene bags or jars, while smaller or delicate organisms can be collected by suction devices. For some cryptic fauna, such as that of macrophyte holdfasts, one approach is to collect the entire habitat, while encrusting or attached organisms can be scraped off into containers. Mobile species can often be induced to leave burrows or crevices by squirting in dilute formalin or bleach.

Apart from the direct picking up of organisms by hand, the simplest collecting by divers is done with a corer on soft bottoms, using a small tube (5-10 cm diameter is popular) of plastic or metal which is pushed or hammered into the sediment, and sealed with a rubber bung before transportation to the surface. Care must be taken (McIntyre, 1971) to ensure that the flocculent material lying on the sediment surface is collected, since this often contains a significant proportion of the smaller fauna, and is easily lost if small diameter cores are used. Another useful piece of hand-held equipment is the suction sampler, which can readily be devised to pick up small or delicate organisms (Tanner *et al.*, 1977).

Divers can also make a major contribution by working along with surface vessels to position and operate gear lowered from ships. A range of hydraulic and air-lift samplers are available which are particularly suitable for such collaborative work (Elephtheriou and Holme, 1984).

Two further activities in which divers can play important parts are survey operations and underwater experiments. When studying pollution from a point source, surveys along transects can be particularly relevant and in areas of hard

bottom divers may offer the best or even the only possibility of obtaining data. The transect can be defined by setting out a non-buoyant line on the bottom. If the seabed is steep the line should be fixed to the bottom, and it can serve additional functions (eg for storing and recovering samples) if buoys and clips are attached along the line (Bailey et al., 1967). Quantitative data can be obtained by swimming along a transect and counting individual organisms within an area defined by the length of a horizontal rod (usually 1 m) held at right angles to the transit line. A more accurate method involves the use of quadrats, within which the organism can be visually assessed or photographed, or all the material in the quadrat can be collected. The quadrat is usually defined by laying a rigid frame on the bottom, and frames of up to 1 m^2 can be used without inconvenience, but the size of the frame required will depend, among other things, on the type of habitat under study. Larkum et al. (1967) required 1 m^2 to differentiate significantly between plant assemblages at 30 and 45 m off Malta.

Finally, divers can make a unique contribution by their ability to set up and service underwater experiments, which can be highly useful in pollution work. Experiments can range from the study of in situ respiration of organisms or communities (Boynton et al., 1981; Loeb, 1981) to the setting up of underwater enclosures for the manipulation of populations and habitats.

In summary, divers can play a major role in ecological studies, but because of the short time they have available underwater, it is important that they should optimize their activity by careful advance planning. In addition, the dangers of their operations must be fully recognized and allowed for.

5.4.5.2 Photography

Photography and television are now widely used in benthic studies and offer the considerable attraction of non-destructive sampling (Holme, 1984). In the intertidal zone photographs, particularly in colour, provide valuable records of the distribution of plants and animals, while aerial photography allows a more extensive coverage and assists in mapping and defining shorelines and submerged reefs and banks.

It is however underwater that photography, video and TV have a special role to play, used either directly by divers or remotely operated. They may be employed in conjunction with other sampling approaches or used as the major aspect of an investigation. For estimating epifauna on hard bottoms or for enumerating large, sparsely distributed organisms these techniques are invaluable, and sometimes provide the only possible means of studying animal behaviour in situ. Also, a photographic or TV survey of an unknown area can be of great assistance in planning a detailed programme.

One very successful technique is to use stereo-photogrammetry (Lundälv, 1971). Here a pair of cameras are mounted on a frame and set to take pictures at fixed distances from the substrate. The stereo pairs of pictures so obtained can then be analyzed in a stereo- comparator and 3-d images reconstructed. This technique is especially useful for subtidal studies where growth rates can be measured, in the third dimension, down to 0.5 mm. Figure 6 shows the frame. Used underwater a bar is attached to the substratum with notches at fixed distances along the bar. The camera frame is hung on the bar and replicate pictures can then be taken at sites along the bar (Christie et al., 1985).

Once photographs have been obtained it is possible with modern techniques to transfer the data directly to the computer. The photograph is placed on a digitizing board and then using commercially available software the community can be sampled, either under fixed points (the point sampling method to give areas of coverage) or the areas of dominant species can be traced using the digitizer. The data are stored directly in the computer for further analyses of spatial and time series changes in abundance patterns of individuals and species.

Detailed studies of growth rates of individual species can be made, provided the exact same area is sampled at each time interval. The photograph is placed on the digitizing board and from the coordinates given, the computer stores the data for each individual and can be programmed to plot, for example, growth rates from these data. Examples of the use of such techniques can be found in Wethy (1984).

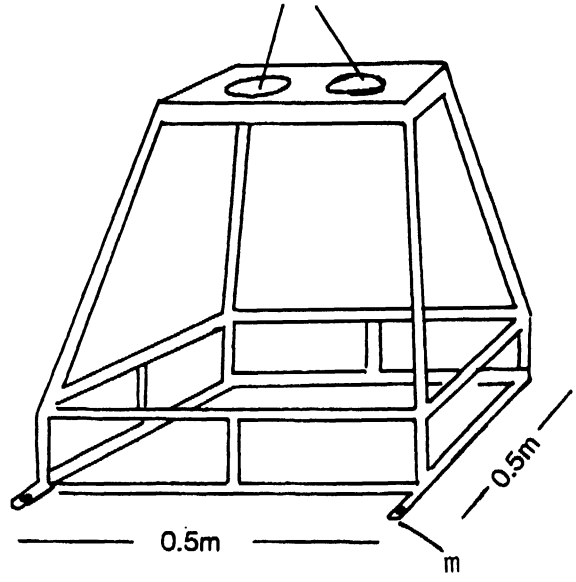

Figure 6. Stereophotographic frame used in conjunction with underwater camera; c- holes for insertion of cameras; m- frame mounting point on bar bolted to study site (from Lundälv, 1971)

Technology in this area is expanding rapidly. It is now possible to use cameras at remote sites, which digitize pictures and relay these by radio directly to storage units at the home base. The digitized pictures can be enhanced using software developed for processing satellite images. Automatic recognition of dominant species should be fairly straightforward so that it should soon be possible to register automatically changing dominance patterns at remote underwater sites (Christie, 1983).

A new and important development is the sediment profile imaging camera developed by Rhoads and Germano (1982) for remote ecological monitoring of the sea floor (REMOTS). The principle is that many sea floor processes can be reconstructed from sedimentary and biological features found in the upper 20 cm of the sea floor. The REMOTS camera allows high resolution imaging of these features by means of in situ photography via an optical prism which sections the sediment. The negatives obtained are analyzed rapidly by a computer image analysis system and measurements of grain size, boundary roughness, thickness of dredged material, depth of RPD layer, epifauna,, tube density surface aggregations of bacteria and bioturbation can be measured. The system in its latest development (Science Applications International Corporation, Admiral's Gate 221 Third St., Newport, R.I. 02840, U.S.A.) can be used down to 4000 m and can sample up to 100 sites per day.

5.4.6 Treatment of samples

Samples from dredges or trawls will usually consist of macrofauna relativelyfree of fine sediment, but often associated with coarse material including pebbles and rocks, which should be examined for encrusting organisms before it is discarded. Large organisms, whether attached or free, should be examined first in seawater. Some species (such as actinarians and other soft cnidarians, turbellarians, opisthobranchs and other molluscs without exoskeletons, nemertines, echiurids, priapulids, sipunculids, and enteropneusts) contract on preservation and may radically change their body form. These require to be anaesthetized (using menthol crystals or $MgCl_2$ up to 4% concentration) before preservation (see Steedman, 1976 and Lincoln and Sheals 1979 for detailed discussion of narcotizing agents). Most taxonomic groups, however, can be preserved and stored in the same solution as used for primary fixation (e.g. 5-8% formalin in seawater). To prevent the formalin solution from becoming acidic and dissolving calcareous structures, use a buffering substance such as hexamine (about 8g hexamine per litre of 2% formalin solution). Sponges and halothurians should be stored in 70-80% alcohol.

Grab or core samples, on the other hand, will be obtained usually in a large volume of sediment and must be processed before preservation. Macrofauna and meiofauna require different approaches.

5.4.6.1 Macrofauna

Initial separation of the organisms from the sediment in the field is usually done by sieving through a screen, after which the residue on the screen is transferred to sample jars, preserved and labelled. Further separation from any remaining sediment ('sorting') prior to final identification and processing is done in the laboratory.

Screens are made from high quality stainless steel or bronze gauze at the bottom of stainless steel or plastic frames 15 to 25 cm high, depending on the sieving procedure to be applied. The free surface of the screens should be about 1,000 cm^2, or 30 x 30 cm; the outer surface must be reinforced, for instance, by a stainless steel cross. If using a series of screens it is convenient to construct frames in the form of drawers to be placed in a rack-like stand. Ideally, they should be made to fit completely into a large plastic or enamel tray so that all the screened material can be shaken down at once from the sieve into the tray.

The total sample or portions are transferred from the grab into the upper sieve and then the sieving is done by washing the material with gentle jets of seawater, shaking by hand and separating agglomerations. Fixed sprinkler-tubes or flexible heads, such as a shower nozzle, must be used for washing. For large sampling programmes, and if working in heavy seas, more robust systems for sieving operations are recommended, such as the Holme's hopper (Holme and McIntyre, 1984) shown in Figure 7. If no running seawater is available, the simplest sieving method is to transfer a portion of a sample into a sieve or tightly connected series of screens placed in a fairly large bucket with seawater, and to shake continuously until the sediments are washed out.

It should be stressed, however, that all procedures described above may damage delicate organisms, particularly polychaetes. Therefore, Sanders extraction methods (Sanders et al., 1965), are recommended for high-level sampling programmes (Figure 8). The sample is washed by putting it in a large container which has a spout near the top, much like a coffee pot. A large diameter water hose (e.g. 4 cm) is pushed down into the sediment, and a large volume of water running at a low velocity is pumped through the sediment. The resulting suspension of animals and fine-grained sediment pours out the spout and then through the mesh screen. The animals are retained on the screen. Large animals are immediately picked out and preserved. At the end of the washing process there are three fractions: animals taken out, the fauna retained by the screen and a coarse fraction remaining in the container, consisting of coarser sediments (heavier organisms such as molluscs). The three samples are preserved separately. This method is time consuming but it is also extremely gentle, and in general the animals are well preserved and relatively undamaged.

The mesh size of the screen used will be determined by the purpose of the investigation and the type of sediment encountered. Usually a mesh of 1.0 mm - 0.5 mm will be required, but smaller meshes may be used with very fine sediments or when juvenile stages of the fauna are required.

When sorting preserved samples, it should be remembered that formalin is toxic and probably carcinogenic. It should therefore be handled with great care and a means of waste air exhaustion should be provided for all laboratory procedures. For sorting, the samples should be washed thoroughly with tap water to ensure that sorters are not exposed to formalin vapour. Sorting may be facilitated by staining the sample first, especially if many small animals are present. Rose Bengal is a suitable stain and the following procedure is recommended (ICES, 1990):

- wash the sample free from the preservation fluid by using a sieve with a mesh size smaller than 0.5 mm.

- allow the sieve to stand in Rose Bengal stain (1 g/dm^3 of tap water + 5 g of phenol for adjustments to pH 4-5) for several minutes with the

sample well covered.

- wash the sample until the tap water is no longer coloured.

If biomass determinations are required, this can be done using wet weight, dry weight or ash-free dry weight, from either fresh or fixed material. For more detailed work, energy content or equivalents of carbon, nitrogen or phosphorus may be determined, but fresh material only should be used for these measurements.

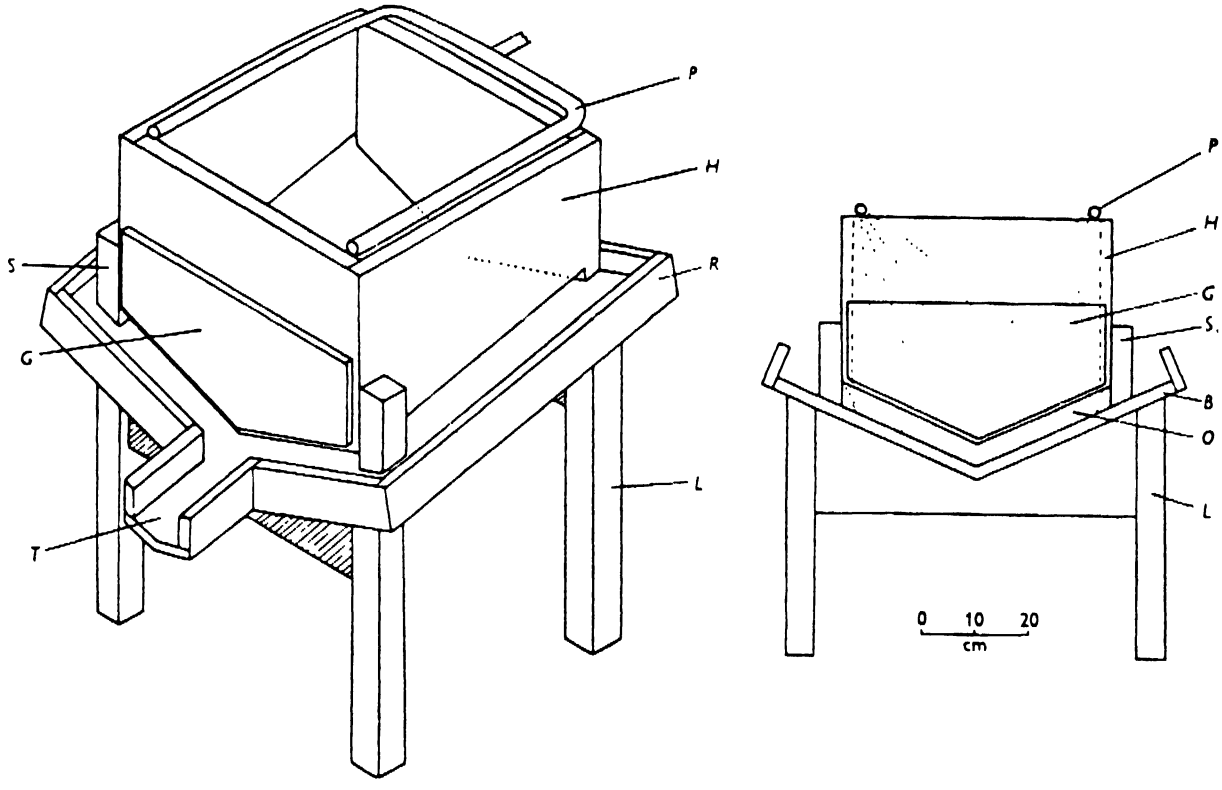

Figure 7. Holme's hopper for sieving benthic samples. P - pipes supplying jets along top of hopper; H - side-wall of hopper; R - retaining wall wall at side of base (B); T - spout; G - rising gate; S - short legs supporting hopper off base; L - legs; O - sediment seen through gap between hopper and base. (From Holme and McIntyre, 1984 with kind permission of Cambridge University Press, Cambridge)

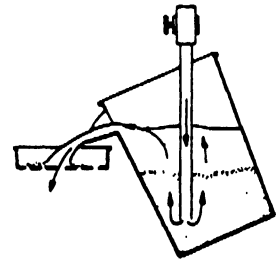

Figure 8. Overflow elutriation system. (From Sanders et al., 1965, with kind permission of Pergamon Press Ltd., Oxford)

Fresh wet weight is to be preferred to formalin wet weight, but if the latter has to be used, weighing should not be done until at least three months after fixation (Brey, 1986).

The wet weight is obtained by weighing after external fluid has been removed on filter paper. The animals are left on filter paper until no more distinct wet traces can be seen. Shelled animals are generally weighed with their shells, the water should be drained off bivalves before weighing. When shell-free weights are given, the shell weight should be included in the data list. Echinoids should be punctured to drain the water before blotting on filter paper. As soon as the non-tissue water has been removed, the organisms are weighed with the accuracy required (for adult macrofauna weighing to 0.1 mg is often sufficient). In case tube-building animals have to be weighed together with their tubes, appropriate correction factors should be established. Dry weight should be estimated after drying the fresh material at 60°C, or by freeze-drying, until constant weight (at least 12-24 hours, depending on the thickness of material). Dry weights obtained by lyophilization (freeze drying) are slightly higher than those obtained by oven drying. For Mytilus, lyophilized tissues weighed 10.9% more than oven dried tissues (Gaffney and Diehl, 1986).

The use of ash free dry weight is recommended in routine programmes, since it is the most accurate biomass measure (Rumohr et al., 1987; Duineveld et al., 1987). However, it destroys specimens, and the consequences of this should be carefully considered. Ash free dry weight should be estimated after measuring dry weight. It is determined after incineration at 500° C in an oven until weight constancy (ca. 6 hours, depending on sample and object size). The temperature of the oven should be checked with a calibrated thermometer, because there may be considerable temperature gradients (up to 50° C) in a muffle furnace. Caution is advised not to pass a certain temperature (<550° C) since then a sudden loss of weight may happen due to the formation of CaO out of the skeletal material of many invertebrates ($CaCO_3$). This can reduce the weight of the mineral fraction by 55%. This decomposition occurs very abruptly and within a small temperature interval (Winberg, 1971).

Before weighing, the samples must be kept in a desiccator while cooling down to room temperature after drying, as well as after removal from the muffle furnace.

To estimate biomass from length or size measurements conversion factors may also be used (Rumohr et al., 1987; Brey et al., 1988).

The above account of weighing procedures is drawn from ICES (1990).

5.4.6.2 Meiofauna

Extraction, separation and sorting of meiofauna, particularly if needed for reliable quantitative investigations, present a difficult task, and the most time-consuming part cannot be done with the naked eye: all operations, except the extraction, must be performed under a binocular dissecting microscope. The most recent accounts are given in Holme and McIntyre (1984) and in the comprehensive Introduction to the Study of Meiofauna edited by Higgins and Thiel (1988).

The samples taken from a substrate of homogeneous fine sand or mud sediments can be treated relatively easily by the Swedmark method, illustrated in Figure 9. The sample, stirred to break up lumps, is placed in a large vessel and covered with 1 to 2 cm of seawater. The mud surface is then pumped into suspension, using a large silicone- coated pipette, and transferred to a nylon sieve or series of sieves (250μ, 62μ), the largest having a diameter slightly less than the normal size of the petri dish used. Sieving is done by gently rocking the sieve in seawater, either in another vessel or in the original one so that the filtrate is returned to the original sample. When this is complete the sieve is placed in seawater in a petri dish so that the fauna can be examined under a binocular microscope before being transferred from the sieves, when they are likely to be damaged.

For meiobenthic samples of coarser and sorted sand (true interstitial meiofauna) the Boisseau elutriation method in closed- system is recommended (Figure 10). This method is more sophisticated but not time-consuming. The

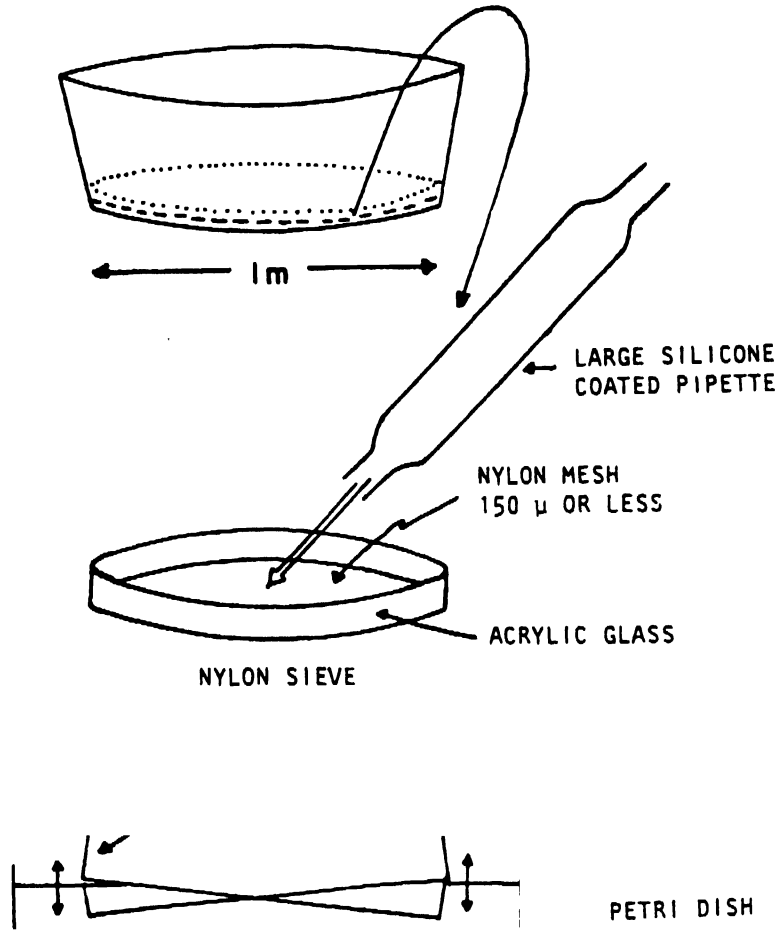

Figure 9. Swedmark method for extraction of meiofauna. (From Hulings and Gray, 1971, with kind permission of Smithsonian Institution Press, Washington)

sample is placed in the separation funnel and an equal volume of 6% $MgCl_2$ solution is added (for anaesthetization of organisms which tend to attach to sand grains). After about 10 minutes, a continuous stream of filtered seawater is introduced through the tap on the separation funnel. After 15 minutes of elutriation, the tap on the tube above the filter is opened and the water allowed to drain through the sieve (50 to 70μ mesh). The sieve is inverted in a petri dish and the meiofauna washed off with a jet of filtered seawater. A large part of the light fauna will be collected on the sieve, but heavier organisms, such as molluscs, ostracods and foraminifers, might remain in the sediment residue so this has to be examined microscopically. This method can also be satisfactorily used for the elutriation of preserved samples; in this case an open-system of a continuous stream of seawater can be applied, only the incoming seawater must first pass a filter in order not to contaminate the sample.

For the treatment of very heterogeneous samples, such as those obtained on hard or marl-detritic bottoms, a convenient, although not entirely quantitative, method is the seawater-ice technique described by Uhlig et al. (1973) (Figure 11). The sample is placed at the lower end of a large plastic tube tightly covered by 120 to 150μmesh nylon gauze, which just dips into filtered seawater in a collecting dish. The sample is covered by a layer of cotton wool, and the tube is filled with the crushed seawater ice. As the ice melts, motile meiofauna move through the gauze into the collecting dish due to salinity/temperature gradients and the streaming action of the water of different densities. If the samples to

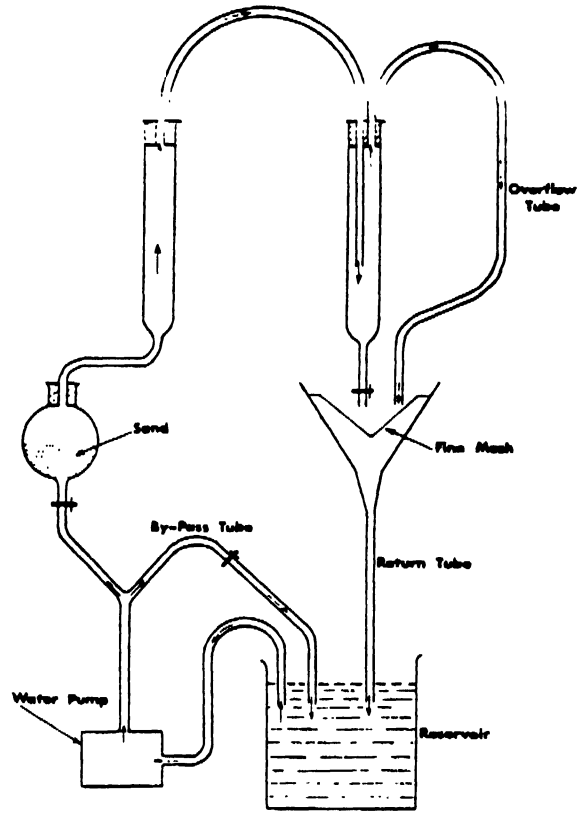

Figure 10. Boisseau type apparatus for elutriation of meiofaunal samples, closed-system arrangement. (From Holme and McIntyre, 1984, with kind permission of the International Biological Programme, London)

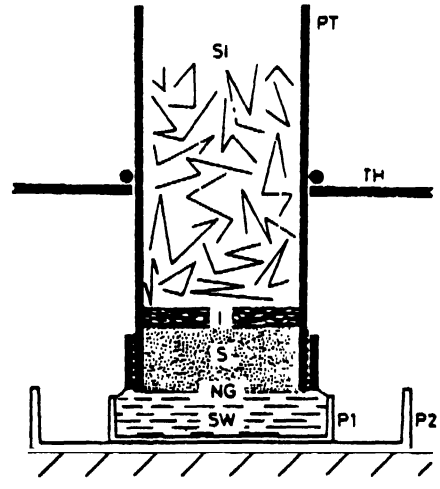

Figure 11. Uhlig's method for the extraction of meiofauna I - insulation material; NG - nylon gauze; P1, P2 - petri- or culture dishes; PT - plastic tube; S - sediments; SI - seawater ice; SW - sea water; TH - tube holder. (From Uhlig et al., 1973, with kind permission of Biologische Anstalt Helgoland, Hamburg)

be treated contain a significant amount of mud, silt or clay, it is advisable to wash them on a 50 to 70μ screen before this treatment. Obviously, only living samples can be processed by this method.

A similar principle but using light can be used for the extraction of phototactic vagile meiofauna inhabiting seaweed. Clumps of algae are placed in a glass jar with seawater and exposed to a strong light source (sun or lamp) from one side only. The creatures assemble along its illuminated surface and can be picked up by a pipette. Subsequent washing of the algae clumps and examination with a low silver microscope is recommended for quantitative results.

A colloidal silver polymer (Ludox-TM) can be used to separate meiofauna from sediment and debris. The standard technique is described by De Jong and Bouwman (1977) and a more rapid method involving centrifugation is outlined in McIntyre and Warwick (1984).

As mentioned, the final separation and sorting of meiofauna can be done only under a stereoscopic microscope. The best type of sorting vessel is a medium-size petri dish, with marked lines on its outerbottom for better orientation while scanning the surface covered by the sample. For separation of organisms, capillary pipettes, fine needles, loops and watchmaker's forceps are needed. In order to make them more clearly visible, and to differentiate biota from detritus and sediment particles, treated samples should be stained with Rose Bengal after being preserved. For this purpose 10 ml of the stock solution (1 g stain powder/100 ml ethanol) is added to 100 ml of sample plus preservative.

5.5 Analyses of results

Methods of analysis for data obtained from both hard and soft substrata are similar and there are no special methods that need to be applied separately. With the widespread availability of powerful personal computers and sophisticated statistical analyses in packages it is assumed that such facilities are generally available.

Many packages exist which are suitable for statistical analyses. For PCs, STATGRAPHICS, SAS, SPSS and SYSTAT are highly sophisticated general statistical analysis programmes and relatively easy to use. However, in analyzing species/site matrices for possible pollution- induced effects the best results are usually obtained using a combination of, first, multivariate analysis, and then the statistical programmes testing specific hypotheses. As yet there are no generally available multivariate analysis packages, but one is under development by Clarke and Carr of the Plymouth Marine Laboratory, UK, for the IOC/UNEP/IMO Group of Experts on Effects of Pollution (GEEP) and many of the analyses mentioned below were performed using this programme on a PC with hard disk, Hercules card and math co-processor.

Whereas most biologists have some knowledge of basic statistics and analysis of univariate data (e.g. diversity indices), multivariate methods may be less well-known. In multivariate analyses the complete data matrix of numbers (or/and biomass) of individual species at all stations are analyzed as a single data set. Powerful methods exist, now adapted to PCs, and can be generally recommended as having been shown to be the 'best' method of unravelling the complex effects of pollutants on marine assemblages. Most multivariate analyses, however, have been done on the fauna of soft sediments and the examples given are from this area. Attention is drawn to methods that are likely to be different.

The raw data obtained from surveys will usually be in the form of two matrices of sites and species, one of abundances of the individual species over sites and the other of biomasses of the individual species over sites. The environmental data for sites should be recorded in a similar matrix.

It is recommended that the user of this manual becomes familiar with common computer-based data file systems such as Lotus 123 or DBASE IV. The raw data should be entered in the format of the above-mentioned programmes so that transfer to statistical analysis packages later is straightforward.

The following analysis protocol has been developed over a number of years by many investigators (eg. Field *et al.*, 1982), but was formally described as the

result of a workshop conducted in the Oslofjord, Norway, under the auspices of GEEP and is fully described in Gray et al. (1988):

(i) Multivariate statistical analyses are used to discriminate between sites based on their faunal (or floral) attributes using classification, ordination and discrimination tests.

(ii) Univariate methods are used to determine levels of disturbance or 'stress' at given sites.

(iii) Correlation of (i) and (ii) above are tested against measured pollution levels.

(iv) Experimental investigations are made testing cause and effect relationships.

5.5.1 Discrimination between sites

Two basic techniques are used to discriminate between sites, namely ordination and classification. An ordination attempts to present a picture of the relationships between samples in terms of their similarity in species abundances or biomass. In the, usually 2-dimensional, picture the relative distance apart of any pair of samples reflects their relative dissimilarity. Cluster analysis, by contrast, forms groups of samples where samples within groups have more similarities than those in separate groups. The methods are complementary and are often plotted together to indicate the degree of similarity in the groupings obtained.

Before using multivariate methods the data are usually subjected to transformations which change the dominance weighing of species. The most widely used transformation is that of $\log_{10}$ (n + 1) but here a less stringent transformation $\sqrt{\ }$ is used. To illustrate the degree of stringency of a transformation, for 100 individuals per sampling unit the transformed value becomes:

$$\sqrt{100} = 10, \qquad \sqrt{\sqrt{100}} = 3.16 \qquad \log_{10} 100 = 2$$

There are a large number of different ordination methods and most are generally available as packages on main-frame computers. Some of the most widely used are Principal Components Analysis (PCA), Principal Coordinates Analysis (PCoA), Multi-dimensional Scaling (MDS) and Reciprocal Averaging (RA) and its variants such as Detrended Correspondence Analysis (DECORANA). Clarke and Green (1988) and Warwick and Clarke (1991) give a description of these methods in relation to their application to pollution studies, and should be consulted for further details. In general the preference by practicing ecologists is to use MDS or DECORANA rather than the computationally simpler PCA and PCoA.

Clustering methods either fuse similar stations into larger and larger groups, so-called agglomerative methods, or divide one group into smaller and smaller dissimilar groups, so-called divisive methods. There is a wide choice of indices on which to cluster. There is much merit in attempting to standardize to a single method and thus here use of the Bray-Curtis coefficient (Bray and Curtis, 1957) followed by an hierarchical, agglomerative method employing group- average linking and the results displayed as a dendrogram are recommended (Gray et al., 1988).

There are statistical methods to determine the significance of differences between replicated community samples in either time or space, e.g. the simulation/permutation test ANOSIM (Clarke and Green, 1988; Clarke, 1990). Likewise methods have been developed to examine the species that distinguish between the site groups such as TWINSPAN (within the DECORANA package) or SIMPER a programme developed for use with MDS analyses by Carr and Clarke (unpubl.) of the Plymouth Marine Laboratory, U.K.

The range of multivariate analysis techniques available is large. Thus by changing various aspects of the technique, a wide range of results can be obtained. There is therefore, much merit in trying to standardise the techniques used so that comparisons between localities of widely differing geographical

regions and pollutants can be made. To this end, suites of standardised analyses methods are to be preferred. Many authors use DECORANA and TWINSPAN and/or a recently developed, powerful and easy to use PC based programme, PRIMER by Carr and Clarke of the Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH.

Figure 12 shows sites along a heavily polluted gradient in the Frierfjord/Langesundfjord area of Norway, hereafter called Frierfjord, (the chemical gradient is described in Abdullah and Steffanek, 1988). Figure 13 shows the multivariate analyses, classification and a MDS ordination. On both analyses, sites A, E, and G are clearly separated from each other and from the group B, C and D. Figure 14 shows data for DECORANA and RA showing similar findings. Thus sites are separated but there is no indication of whether or not this separation can be related to pollution.

Figure 15 shows classification and ordination analyses of macrobenthos sampled around an oil platform in the North Sea in 1987, (Gray et al., 1990). There are four clear groupings of sites A, B, C, and D. Again site groups are separated clearly but whether or not this is due to pollution can only be ascertained from other analyses (see later).

Bellan and Bourcier (1990) show the application of similar techniques to the benthos near Marseille suffering from organic enrichment from sewage waste and these authors should be consulted for details of effects relevant to Mediterranean fauna. Likewise, Zenetos and Papathanassiou (1989) have used the PRIMER programme and multivariate analyses to show effect of tannery waste on the benthic fauna in the Aegean Sea.

5.5.2 Univariate methods determining levels of disturbance

The multivariate methods simply separate groups of sites that have similar faunal groupings and do not give any indication of the underlying causes of species differences between sites. In order to investigate whether or not pollution effects are involved, univariate methods are used. These methods fall into two categories, those which must refer to unpolluted control sites and those which can determine whether or not an individual site is affected.

5.5.2.1 Between site comparisons

Species, abundance, biomass. The simplest method to compare sites is to plot the number of species, abundance and biomass - SAB (Pearson and Rosenberg, 1978) and ratios of B/A and A/S. Figure 16 shows plots for sites A to G using the earlier data set, Figure 13. The plots show that sites A and G have highest B/A and lowest A/S ratios suggesting that these sites are relatively undisturbed whereas sites B, C, D and E are intermediate.

Diversity. It has long been a tradition in pollution monitoring that diversity indices are used to assess whether or not particular sites are polluted. This probably arose because pollution control authorities were often engineers who were not familiar with biological complexity and simply wanted an index which integrated both number of species and individuals per species. Diversity indices are supposedly high at unpolluted sites and low at polluted sites. However, in general, statistically significant reductions in a diversity index are accompanied by such profound changes in the biological system studied that the method is a rather poor indicator of pollution-induced change (Bayne et al., 1988).

There is a wide variety of diversity indices in common usage, all of which are highly correlated between themselves. The most widely used diversity index is undoubtedly the Shannon-Wiener index, which is recommended here:

$$H' = - \sum_{i=1}^{s} p_i \log p_i$$

where $p_i = n_i/N$, s = total number of species, N = total number of individuals n_i = number of i the species from 1 to s, (Shannon and Weaver, 1949).

The base of the logarithm used can vary. Some people prefer to use the base

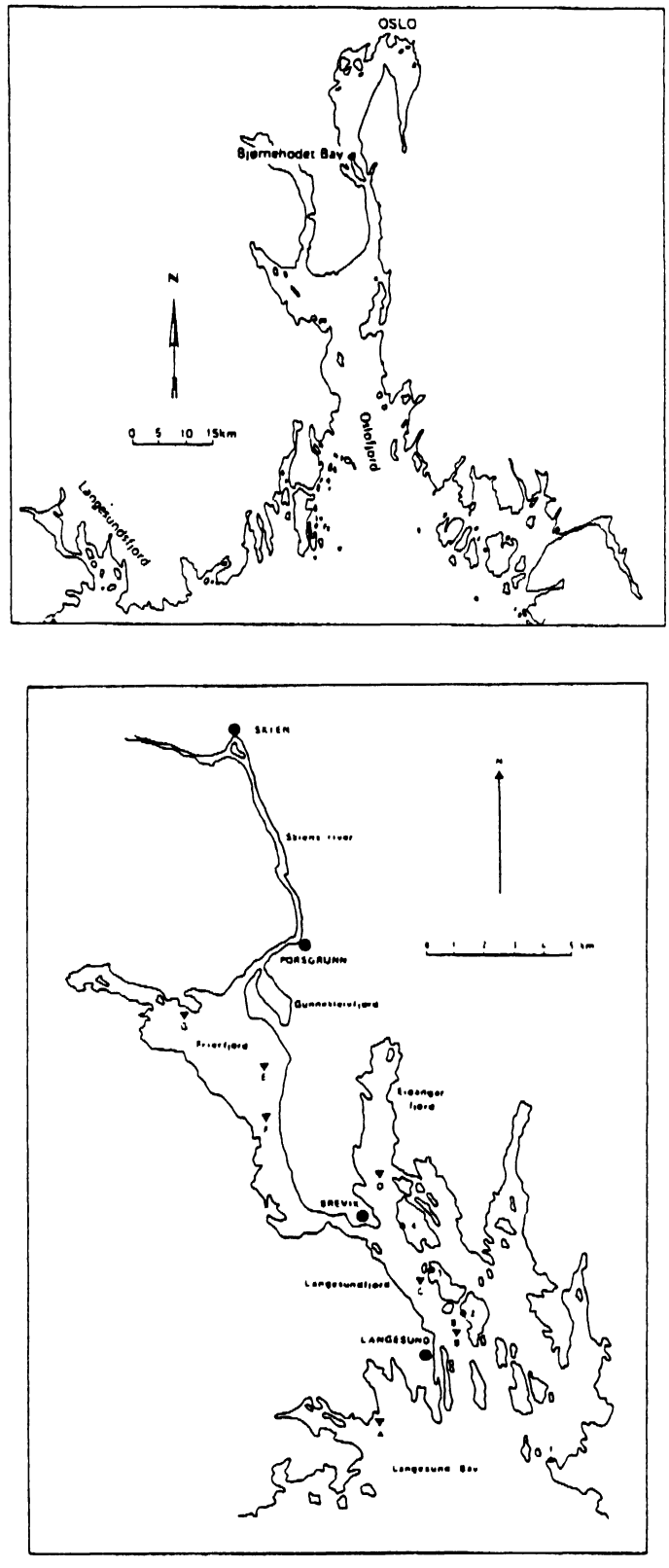

Figure 12. Sites along a gradient of pollution at Frierfjord/ Langesundfjord, outer Oslofjord, Norway, at which analyses of benthic communities shown in Figs 13 and 14 were conducted [see Gray et al. (1988) for details]

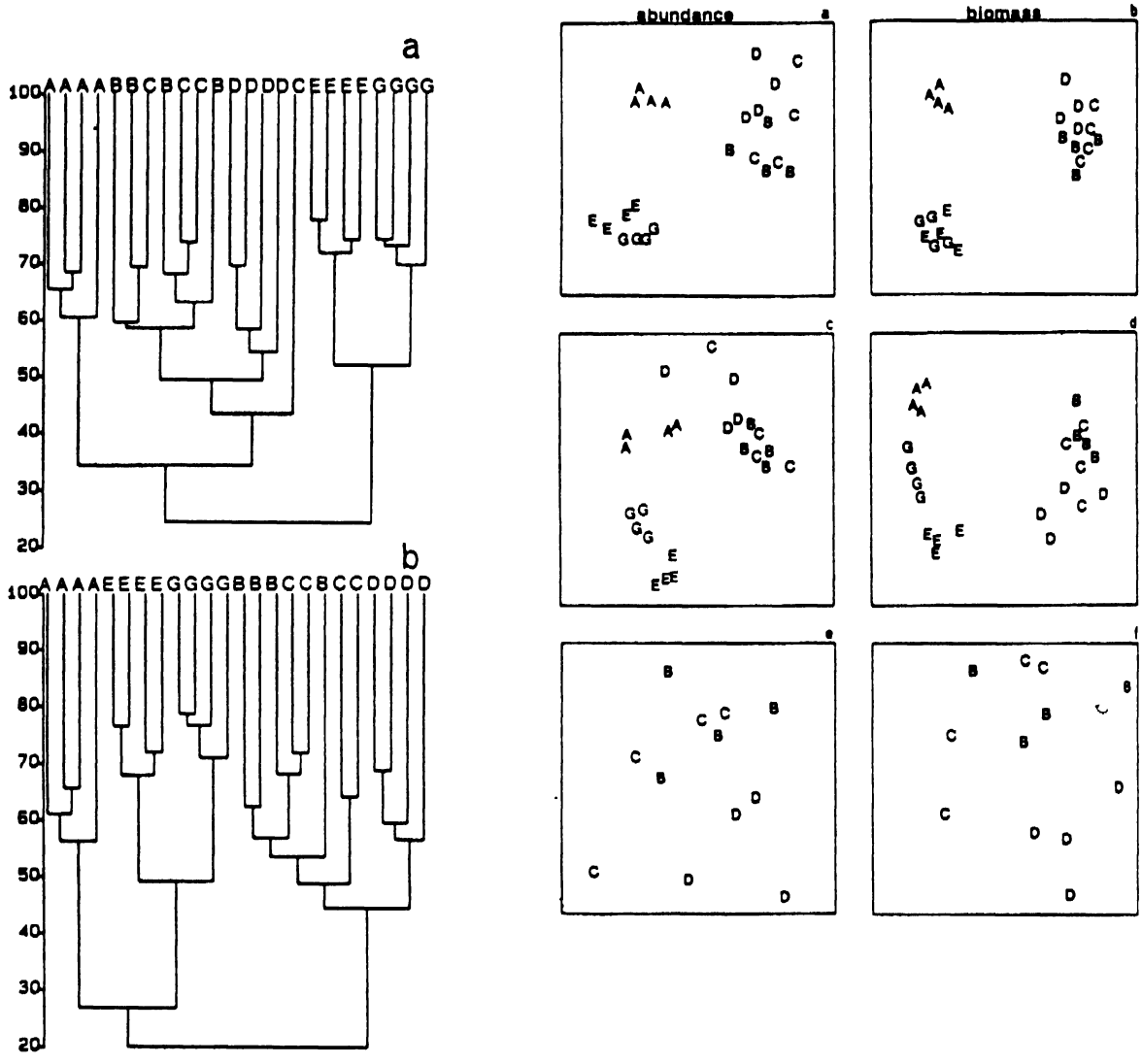

Figure 13. Multivariate analyses of benthic communities at Frierfjord/ Langesundfjord. (Left) Classification analysis showing dendrogram for group-average clustering of Bray-Curtis dissimilarities (y-axis) between 24 field macrofauna samples (x-axis), consisting of 4 replicate grabs at each of sites A to E and G. (a) Species abundance data after √√ transformation (b), Species biomass data after √√ transformation. (Right) Multidimensional scaling (MDS) ordination based on Bray-Curtis similarities between grab samples. (a) √√ abundance (b) √√ biomass (c) raw abundance (d) raw biomass (e) √√ abundance sites B to D only (f) √√ biomass sites B to D only (from Gray et al., 1988)

2, that of the original formulation, but other bases e.g. $\log_e$, or $\log_{10}$ are equally valid. Care should be taken however, in comparing diversity indices in that the base used is both stated and similar.

Frontier (1985) has given an extensive review of diversity and related properties in aquatic ecosystems and should be consulted for a detailed discussion of this topic.

Another facet of the diversity concept which is not covered by the index and which is often quoted is evenness (Pielou, 1966):

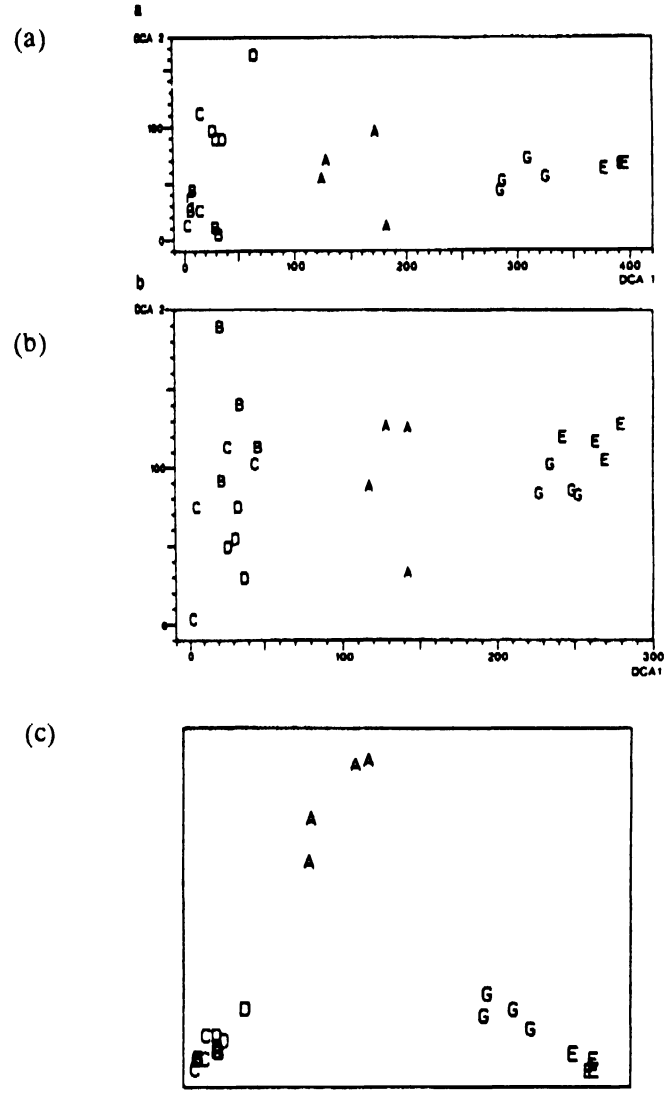

Figure 14. Detrended Correspondence Analysis (DECORANA) ordination of data from sites in Fig. 12 (**a**) no transformation (**b**) $\sqrt{\sqrt{}}$ transformation (**c**) reciprocal Averaging (RA) ordination of data from sites in Fig. 12 (from Gray et al., 1988)

$$J = H'/H_{(max)}$$

In terms of the above equation $H_{(max)} = \log_2 s$

For the Frierfjord data, plots of diversity and evenness (Figure 16b) show that site E had highest diversity closely followed by site A, whereas B, C and D were lowest. Figure 17 shows data for the Statfjord oilfield in the North Sea with low diversity near the platform at high oil levels in the sediments, and for a mine waste site in Norway, again with low diversity near the outfall.

Although Hill (1973) calculates a range of 9 different diversity indices ranging from the number of species to an index close to evenness there is litle point in calculating them all as there is strong correlation between them all and the Shannon-Wiener index is as good as any other index.

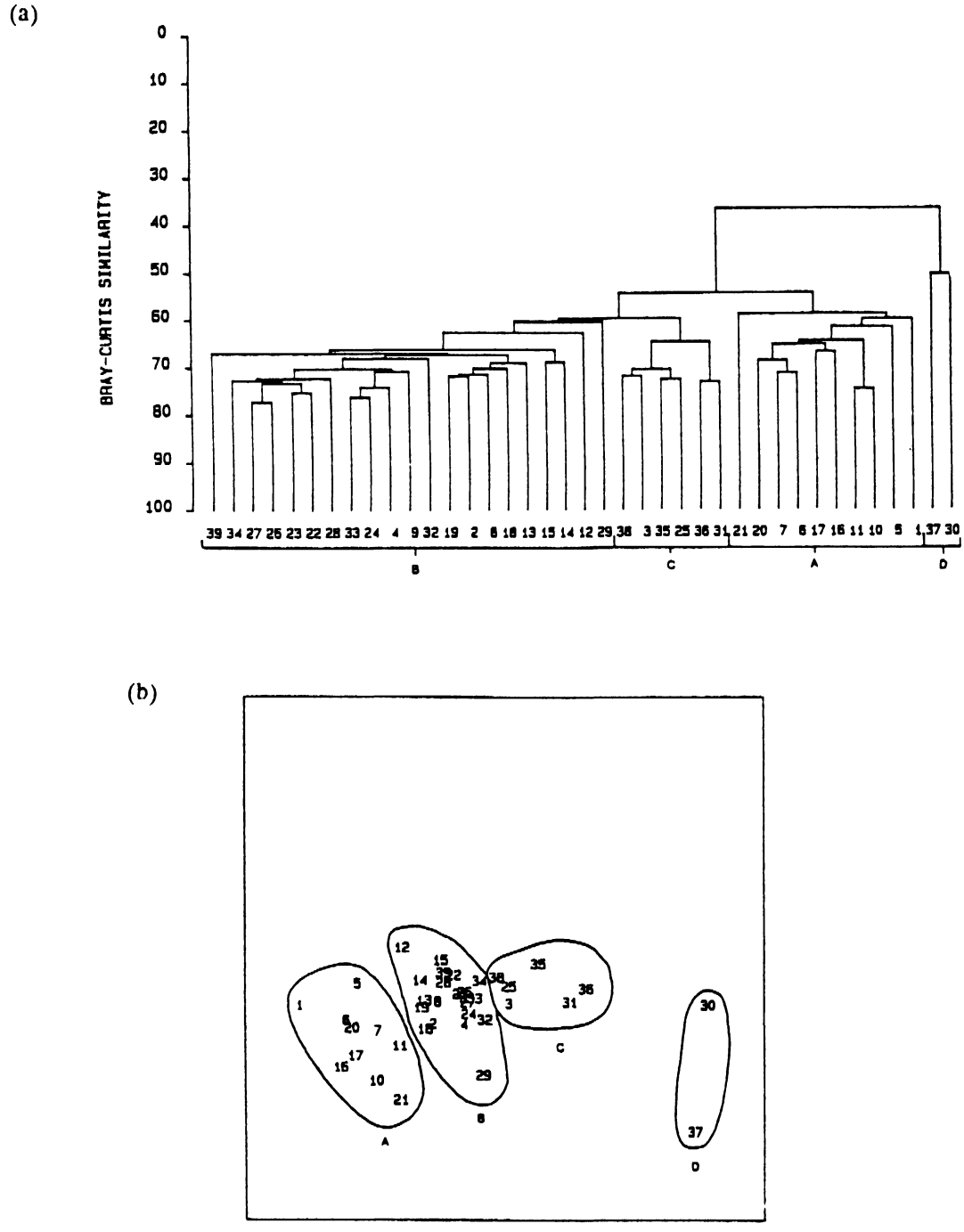

Figure 15. a) Classification analysis of benthic data from Ekofisk oilfield, North Sea in 1987 (√ transformed data with Bray-Curtis dissimilarities). Numbers refer to site numbers and letters to groups of stations separated at 62% dissimilarity, except for group D where two sites are separated at 50%. b) MDS ordination of the same data with superimposed groupings from the classification analysis in (a). (From Gray et al., 1990)

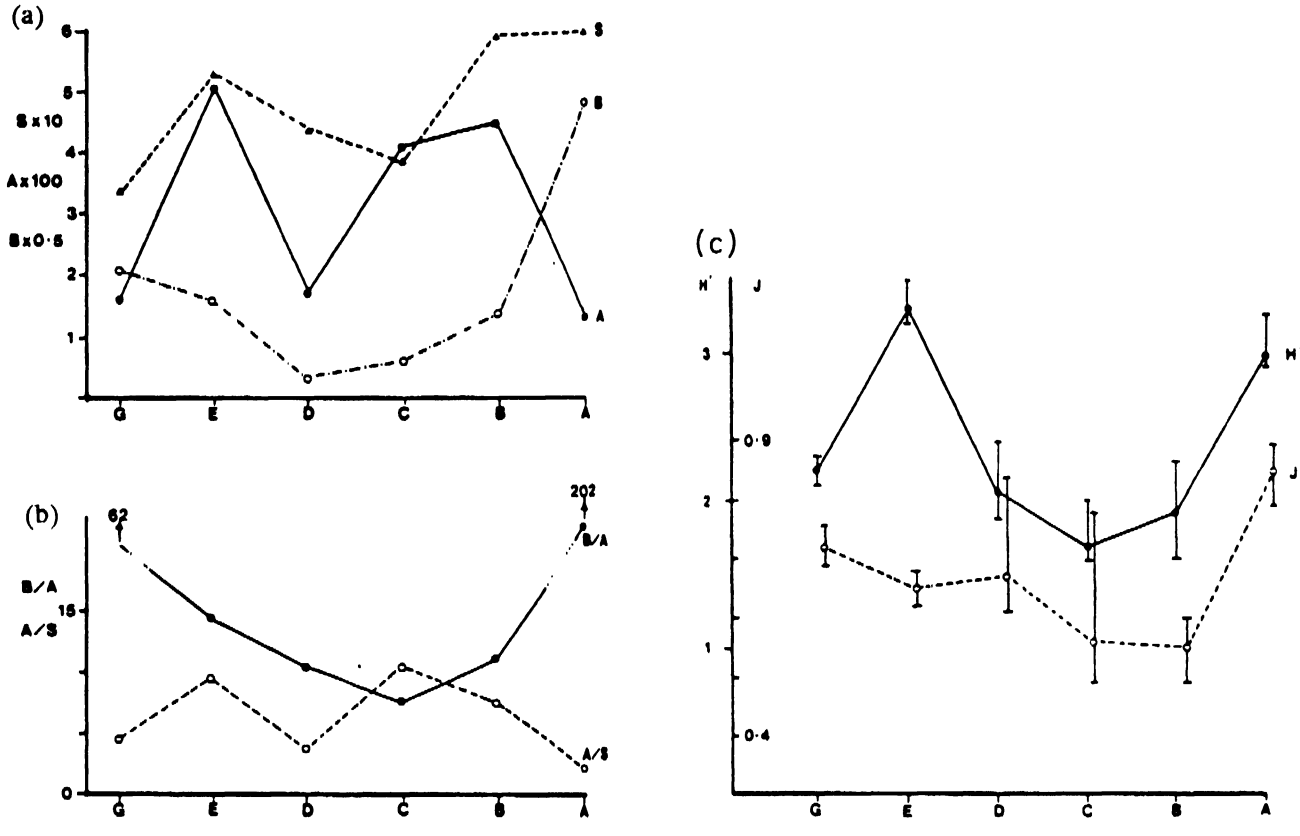

Figure 16. Plots for sites A to G from Figure 12 (a) total number of species (S), mean abundance per 0.1 m^2 (A), mean biomass in mg per 0.1 m^2, (b) ratios of B/A and A/S across sites, (c) Diversity (H') and evenness (J) across sites. (For details see Gray et al., 1988)

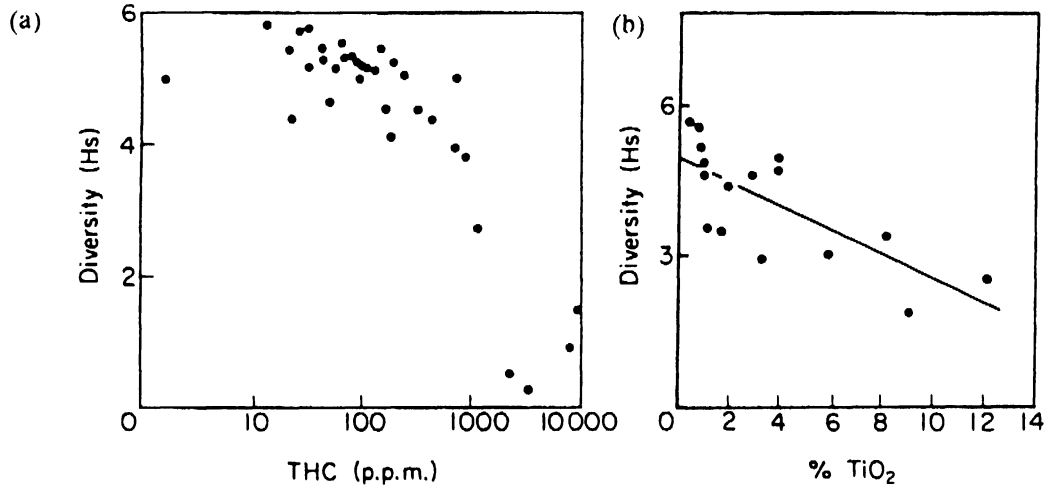

Figure 17. Relationship between species diversity and total oil concentration in ppm for (a) Statfjord oilfield, Norwegian sector and (b) diversity and TiO_2 content of sediment at site of mine waste discharge in Jossingfjord, Norway (from Gray, 1989)

5.5.2.2 Determination of disturbance at individual sites

Two methods have been suggested for assessing whether or not individual sites are disturbed. The first is to plot the number of individuals among species in geometric classes of abundance (Gray and Pearson, 1982). An undisturbed site will have a steep curve, cover few geometric classes and have an abundance of rare species represented by one specimen. A disturbed site will have a shallow curve, which is often disjointed, cover many geometric classes and have few rare species. Figure 18a shows a plot for the Frierfjord data showing site A is undisturbed, whereas C and E are disturbed and B and D are intermediate.

The second method is the abundance, biomass comparison (ABC) method of Warwick (1986) and Warwick et al. (1987) using 'k' dominance plots. Here the cumulative dominance in terms of abundance and biomass are plotted against a logarithmic scale of species rank. Figure 18b shows data for the Frierfjord sites where at sites A and G the biomass curves lie above the abundance curves (undisturbed). At sites C and D the biomass curve is well below the abundance curve indicating moderate to gross disturbance, whereas sites B and E are intermediate.

6.5.2.3 Correlation with anthropogenic inputs

Having some indication that site differences are due to disturbance it is important to ascertain whether this is caused by contaminant loadings or not. The assumption is that both environmental and chemical data have been recorded at the sites where faunal samples were taken. In the case of the Frierfjord example used above, replicate cores were taken and a suite of PAH and metal data were analyzed. For the Ekofisk oilfield data (Figure 15) barium is an excellent indicator of anthropogenic activity as it is used in both oil-based and water-based drilling muds. Thus the barium content in the sediment will be used.

For the Frierfjord data using univariate methods (number of taxa, biomass, abundance, diversity etc.) mean biomass was particularly low at sites C and D and sites A and G had the highest B/A and lowest A/S. This suggests that sites A and G are relatively undisturbed whereas C, D and E are disturbed. Diversity (H') showed that A and E had highest values whereas B, C, D and G had similar values. The ABC plots showed A and G as undisturbed with B and E moderately disturbed and C and D moderately to grossly disturbed. With the exception of the diversity data where the pattern for stations E and G differs from the other analyses, all methods suggest that sites A and G are undisturbed with B, C, D and E being disturbed.

A PCA analysis was done on the Frierfjord chemical data and significant differences between sites were found, (Gray et al., 1988). Site A had lowest heavy metal loadings, G the highest and B, C, D and E intermediate. The measured environmental and pollution variables were then superimposed upon the MDS analyses of faunal data (Figure 19).

Neither sediment type (b) nor PAH (d) correlate with the faunal groupings. The B, C, D site group has sediments with both the largest and smallest particles. The E, G cluster has both high and intermediate levels for both metals and PAH. However, depth (a) separates the clusters well and increases in a uniform manner from left to right. Thus although there is possibly an effect of heavy metals the simplest explanation is that depth plays an overriding role on the species composition of the macrofauna. The site closest to the pollution source, G, showed at most slight indication of organic enrichment. These results are important as they illustrate clearly that interpretation of effects of pollutants on marine benthos must take into account the most significant environmental variables, (depth and grain size) otherwise one might risk attributing effects of pollution (heavy metals) where in fact perhaps only depth is significant. These data again illustrate the necessity in sample design of making sure that 'nuisance' variables are adequately covered. Here a revised sampling strategy in the light of the above findings would be to sample at similar depths in the future.

In the case of the Ekofisk oilfield, the data are from similar depths and grain size. Again diversity was not found to be a particularly useful variable as only the grossly polluted sites had lower values than the unpolluted sites.

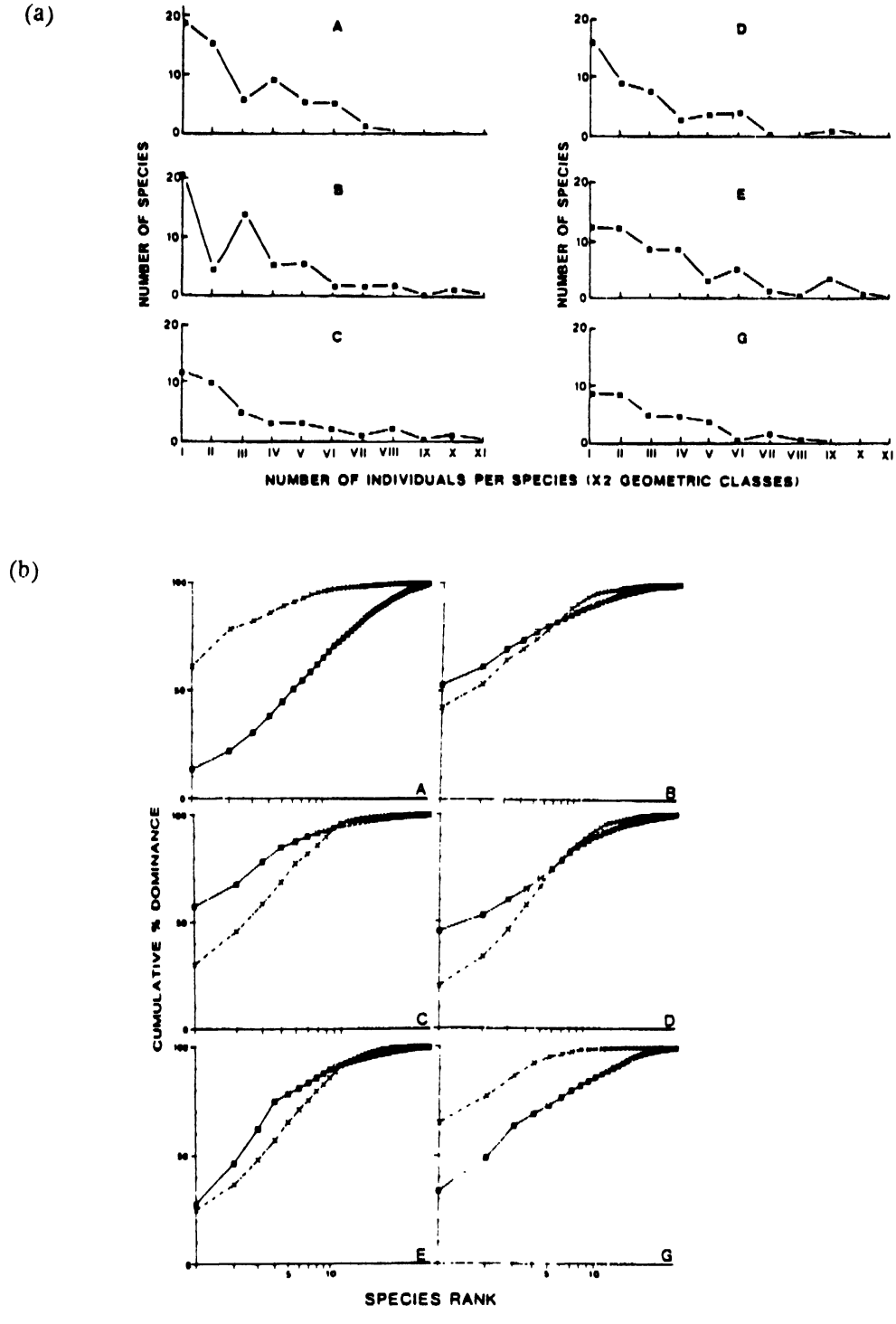

Figure 18. Detection of disturbance at individual sites. (a) Plots of number of species against number of species per individual in geometric classes at sites A to E and G from Figure 12. (class intervals I = 1 individual per species, II = 2-3 individuals per species (III = 4-7 individuals per species etc). (b) Abundance biomass comparison curves (ABC) for sites A to E and G in Figure 12. Abundance squares and solid line, biomass crosses and broken line based on the totals from 4 replicates at each site (For details see Gray et al., 1988)

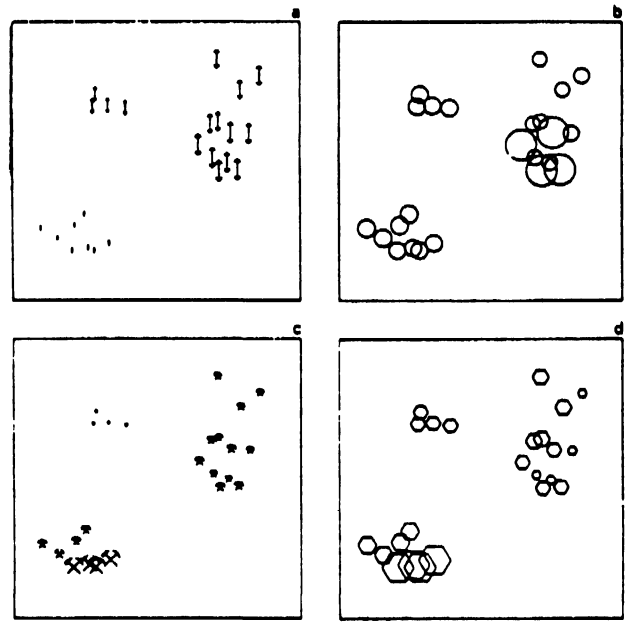

Figure 19. MDS ordination of the 24 field macrofaunal samples from sites A to E and G in Figure 12. The data are shown with superimposed symbols on the original faunal groupings in linear dimensions proportional to the values of the selected environmental variables. (a) water depth, smallest symbol 22m largest 113m. (b) median particle diameter of sediment, smallest 7.8 μm largest 16.5 μm. (c) metal concentration in sediment mean principal component score from PC analysis representing an average of Cu, Zn, Pb, Ni, Cr, Cd levels, smallest -2.9 largest + 3.2 (d) total PAH concentrations in sediment, smallest 4.4 $\mu g\ g^{-1}$ largest 14.8 $\mu g\ g^{-1}$ (Gray et al., 1988)

Figure 20 shows site groupings from the multivariate analyses and the sediment chemistry for the station groupings obtained by the multivariate analyses. Clearly the separation into the non-polluted group A and the just-affected group B can be related to barium content, with a mean of 6.3 at A and 7.6 at B. However, there is no separation between B and C and D on the barium content, all having statistically similar values around 7.6 - 7.8. Yet D has the highest total hydrocarbon content (6.25) which is statistically higher than the other three groups A, B and C. C (mean 7%) is intermediate between both A and B (4%) and D (12%) in the percentage mud content. As the percentage mud probably represents the amount of drilling material deposited, a pollution gradient is reflected from A-D. Thus the Ekofisk example shows clearly how multivariate data can be combined with environmental data and statistical analyses to show the influence of pollutants on benthic communities.

5.5.2.4 Analyses of higher taxa only

One of the most interesting aspects of recent research on effects of pollutants on benthic communities is that effects similar to those shown above can be found when higher taxal levels than the species are used (Warwick, 1988). Using data from the Frierfjord macrofaunal studies Warwick was able to show that analyses done at the level of families of macrofauna gave equally good separation of the faunal groups as that obtained using species (Figure 21). The level of phyla gave less tight clusters but still separated the major station groupings. Similar results were obtained from analyses of the Ekofisk data and for meiofaunal analyses of the Frierfjord data (Heip et al., 1988).

These results suggest that it might be possible to greatly simplify pollution monitoring work by identifying only to the level of family (and possibly only to phylum). Whilst more substantiation of these results are needed, this

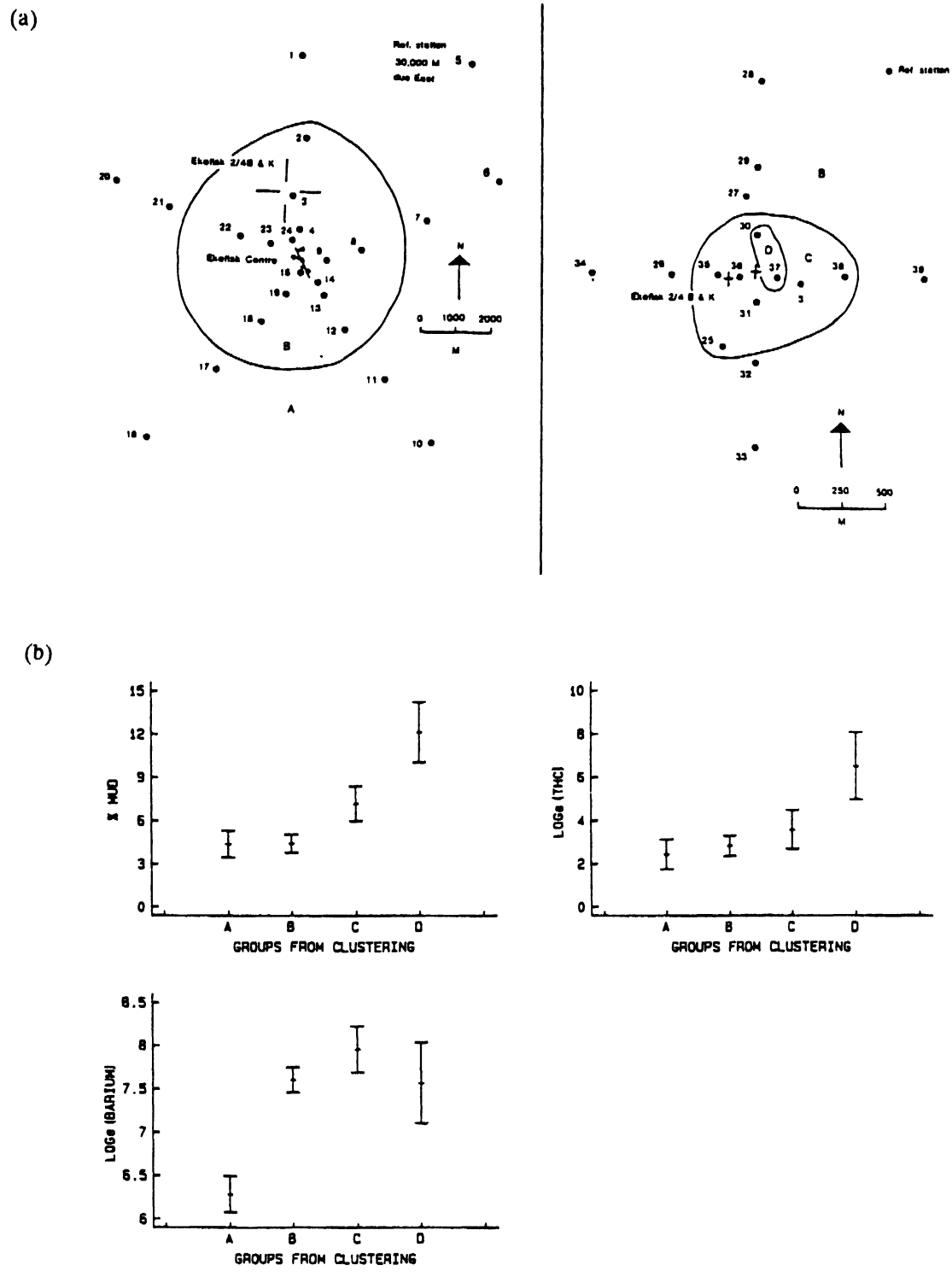

Figure 20. (a) Plots of the site groupings from the multivariate analyses of the Ekofisk macrofauna (Figure 15). Ekofisk 2/4B & K are newly bored holes whereas the Ekofisk Centre is the original oilfield site. The data show 4 groupings with A sites being unpolluted and D being grossly polluted. (b) Plots of selected environmental variables for the site groupings from Figure 15 (Gray et al., 1990)

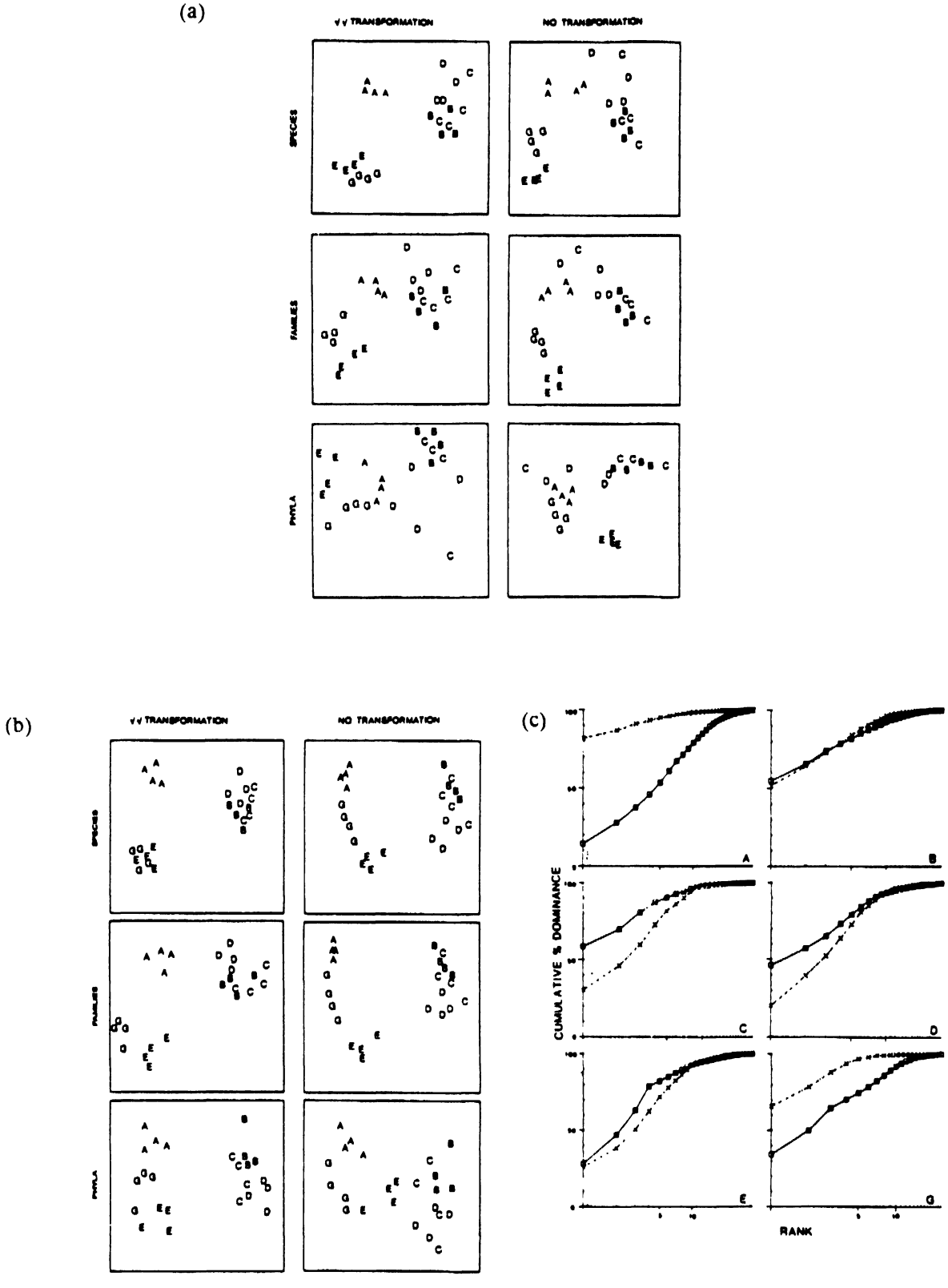

Figure 21. Use of higher taxa in pollution studies. (a) MDS analysis comparing the original species analysis with analyses for families and phyla for abundance data. (b) as in (a) but for biomass data. (c) ABC plots for data using family data (cf Figure 18b for species)[see Warwick (1988) for details]

points the way to possible large savings in costs of benthic pollution surveys. It also suggests that perhaps statistical techniques for analyzing benthic data sets need more research, as ecological theory would suggest that it is species that should best reflect environmental gradients rather than families or phyla.

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APPENDIX 1

Optimal allocation of samples

Samples can be allocated according to the variability of the standard error. To take an example: the area is divided up first according to sediment types using methods similar to those shown above and a preliminary sampling done. The total number of animals found in this preliminary survey taking 7 replicates per station is:

		REPLICATES					
Stratum	Station	A	B	C	D	E	F
1	1	1020	1180	1300	2100	980	900
	2	390	490	210	360	220	310
	3	140	440	360	150	490	1070
2	4	140	150	190	160	150	180
	5	420	950	350	150	180	330
3	6	370	420	700	100	200	190
	7	620	1390	380	450	480	2600
4	8	390	430	110	440	110	180
	9	40	20	350	60	80	20
	10	150	140	660	320	240	880
	11	730	670	470	340	930	370
5	12	1380	1410	1190	2710	1600	1290
	13	1620	320	1550	760	1250	1990
	14	1850	2060	1090	2410	1520	220

Using a statistics package on a PC (e.g. Statgraphics) calculate the means, standard deviation and standard errors for these data, giving:

Stratum	Sample Size (n)	Mean	s.d	s.e
1	21	677.62	504.62	110.12
2	14	260.00	218.32	58.35
3	14	642.14	654.90	175.03
4	21	309.05	381.59	83.27
5	28	1162.86	686.68	129.77
			TOTAL	**556.54**

Calculate each s.e. as a proportion of the Total s.e. and use this proportion to calculate sampling allocation per stratum. Here it is assumed that a total of 65 samples can be taken in the next survey.

Stratum	Proportion of Total s.e.	No. of Samples/Stratum
1	0.198	13
2	0.104	7
3	0.315	20
4	0.149	10
5	0.232	15

It is now possible to calculate how effective this sampling system has been compared with random sampling. First an analysis of variance (using Statgraphics) is ran on the data testing <u>within strata</u> variance compared with <u>between strata</u> variance. This gives:

Source of variation	Sum of Squares	d.f	Mean Square	F Ratio
Between Strata	11902293	4	2975573.3	10.27
Within Strata	26931369	93	289584.6	
TOTAL	38833662	97	400347.0	

The pooled standard deviation within strata s_w is:

$$s_w = 289584.6$$

$$s_w = 538.13$$

Estimated s.e. of Y_{st} = $s(Y_{st})$

$$s(Y_{st}) = s_w / \sqrt{n} \qquad n = 98$$

$$= 538.13 / \sqrt{98}$$

$$= 54.36$$

With purely random sampling $S_y = s / \sqrt{n}$

$$S_y = 400347 / \sqrt{98}$$

$$= 63.91$$

Therefore the stratified sampling reduces the s.e. by

$$(63.91 - 54.36) * 100)/63.91 \%$$
$$= 14.9 \%$$

This is a big change and illustrates the advantages of stratified sampling.

APPENDIX 2

Calculation of number of replicates to be taken

Assume that 10% error is acceptable and call this proportion (0.1)D. The number of samples that should be taken (n) is:

$$n = s^2/ (0.1)^2 x^2$$

$$= 100\ s^2/x^2 \text{ for a 10\% accepted error.}$$

Example: counts = 14,15,12,7,8,14,11,14,10,9,10

$$s^2 = 7.42 \quad x = 11.273$$

First, test whether or not the distribution is <u>random</u>. Using the s^2/x ratio.

If $s^2/x < 1$ the distribution is REGULAR

If $s^2/x = 1$ the distribution is RANDOM

If $s^2/x > 1$ the distribution is AGGREGATED

(Exact tests of these distributions can be found in Elliott, 1971).

Here $s^2/x = 7.42/11.273 = 0.66$ which we accept as random

$$n = 100 * 7.42/(11.273)^2$$

$$= 5.82 \text{ i.e. 6 samples}$$

Most marine species however, are aggregated.

For an aggregated distribution:

Counts: 98,22,72,214,67

$$s^2 = 5202.8 \quad x = 94.60$$

$$n = 100 * 5202.8/(94.6)^2$$

$$= 58 \text{ replicates}$$

This is an enormous number of samples and is clearly impractical. So accept a lower error estimate e.g. 20%.

$$n = 25 * 5202.8/(94.6)^2$$

$$= 14.53 \text{ replicates (i.e. 15)}$$

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